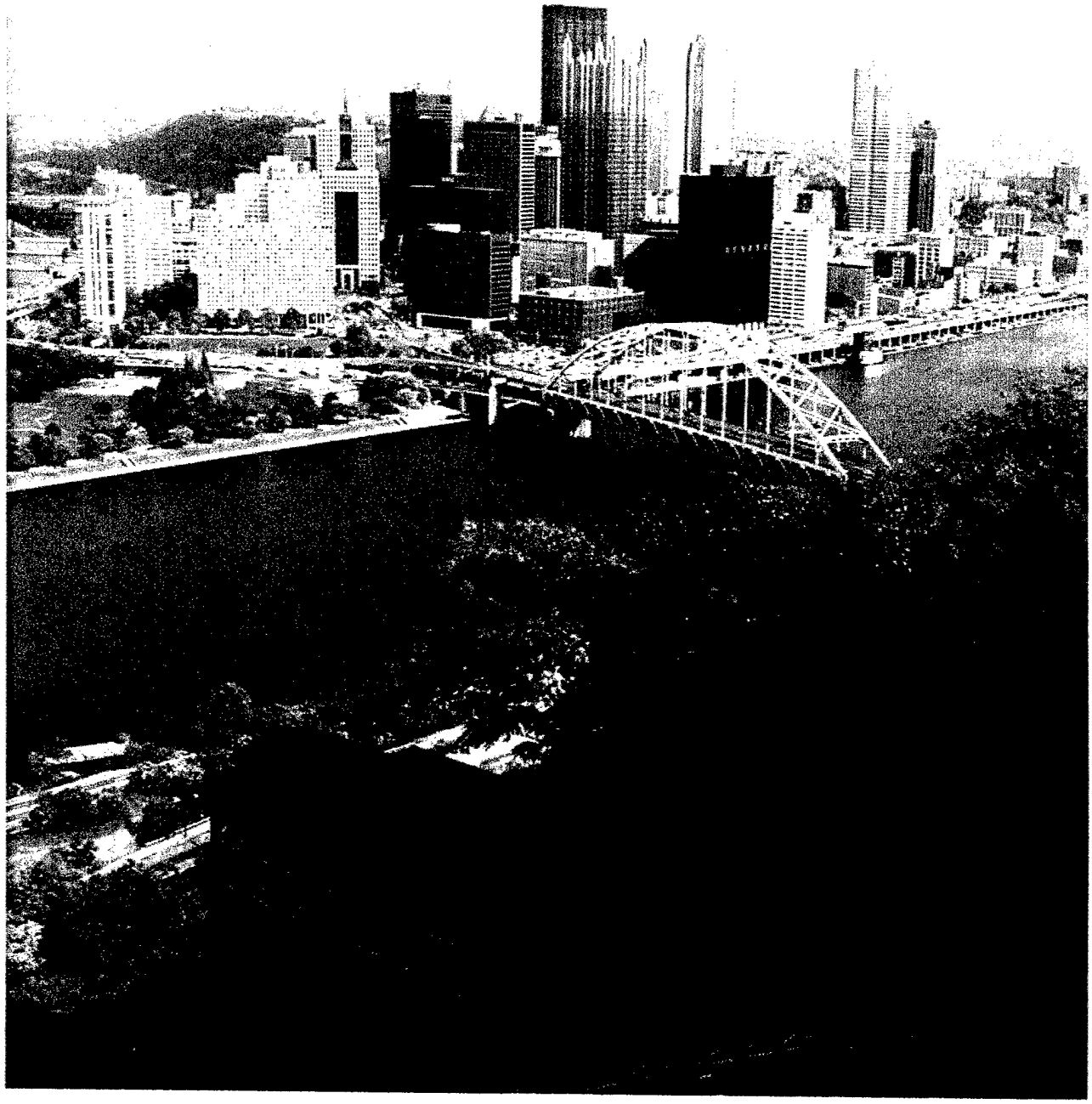
# PLASMID BIOLOGY 2002

*An International Symposium*

June 22-28, 2002

**PITTSBURGH, PA USA**





# *PLASMID BIOLOGY 2002*

## AN INTERNATIONAL SYMPOSIUM

***June 22-28, 2002***
***William Pitt Union***
***University of Pittsburgh***
***Pittsburgh, Pennsylvania, USA***

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# PLASMID BIOLOGY 2002

## *Saturday June 22*

12:00 **Registration**
18:00 **Welcome Reception**
19:00 **Dinner**

## *Sunday June 23*

07:30 **Breakfast at the Union**

08:15 **Opening Remarks: Dr. Arthur S. Levine,** Senior Vice Chancellor for the Health Sciences and Dean, School of Medicine, University of Pittsburgh

**REPLICATION I (Chairperson: Saleem Khan, Pittsburgh)**

08:30 **Keynote Address: Deepak Bastia (Charleston)**
Oligomerization of the yeast replication terminator protein, Fob1p is critical for its ability to arrest replication forks

09:10 **EMBO Young Investigator Lecture: Igor Konieczny (Gdansk)**
Host specific mechanisms for helicase complex formation at the origin of a broad host range plasmid

09:40 **Marcin Filutowicz (Madison)**
Monomers and dimers of π Protein and what they do to make plasmid R6K happy

10:10 **Coffee Break**

10:40 **Dhruba Chattoraj (Bethesda)**
Relaxation of autorepression of the initiator gene of plasmid P1 relaxes the plasmid copy number control

11:10 **Aresa Toukdarian (San Diego)**
A specific region in the N-terminus of the TrfA-44 replication initiation protein of plasmid RK2 is required for recruitment of *Pseudomonas aeruginosa* DnaB helicase to the plasmid origin

11:30 **Rafael Giraldo (Madrid)**
Structural changes in the pPS10 replication initiator, RepA, upon binding to origin DNA

11:50 **Lunch and Departure to Fallingwater OR Pirates Baseball Game**

18:00 **Dinner**

**REPLICATION II (Chairperson: Donald Helinski, San Diego)**

19:00 **Keynote Address: Gerhart Wagner (Uppsala)**
Antisense RNA in plasmids and beyond

19:40 **Sabine Brantl (Jena)**
Antisense RNA-mediated transcriptional attenuation: A comparison between Gram-positive bacteria and *E. coli*

20:00 **George Chaconas (London, Canada)**
Replication of the linear plasmid LP17 from *Borrelia burgdorferi*, the Lyme disease spirochete

20:30 **Coffee Break**

21:00 **Saleem Khan (Pittsburgh)**
Role of the PcrA helicase in the replication of rolling-circle plasmids

21:30 **William Firshein (Middletown)**
Identification of a novel gene product that suppresses the toxicity of a TrfA peptide from plasmid RK2 and its relationship to the DnaA host initiation protein

21:50 **Grzegorz Wegrzyn (Gdansk)**
Replication of lambda plasmids carried out by the heritable replication complex

22:10- **Tateo Itoh (Nagano)**
22:30 Plasmids of the ColE2 family with initiator proteins that synthesize the origin-specific primers

## *Monday June 24*

07:30 **Breakfast at the Union**

**PARTITION/STABILITY (Chairperson: Christopher M. Thomas, Birmingham)**

08:30 **Keynote Address: Stuart Austin (Frederick)**
Time lapse photomicroscopy of P1 plasmid partition in living cells

09:10 **Barbara Funnell (Toronto)**
The interaction of ParA and ParB proteins during P1 plasmid partition in *Escherichia coli*

09:40 **Finbarr Hayes (Manchester)**
The ParFG partition proteins

10:00 **Juan Alonso (Madrid)**
The regulatory protein ω from the broad-host range *Streptococcus pyogenes* plasmid pSM19035: Analysis of binding to operator DNA with one to four heptad repeats

10:20 **Coffee Break**

10:50 **Kenn Gerdes (Odense)**
Prokaryotic analogue of the eukaryotic spindle apparatus

11:20 **Ramon Diaz-Orejas (Madrid)**
The structure of the kid toxin: Localization of functional regions and comparison with the CcdB toxin and with other homologous toxins

11:40 **Monika Oberer (Graz)**
Solution structure and dynamics of the antitoxin ParD from the plasmid RK2/RP4 killing module

12:00 **David Summers (Cambridge)**
ColE1 dimer resolution: How does synaptic complex structure prevent catastrophic dimer formation?

12:30 **Lunch**

**TRANSFER I (Chairperson: Manuel Espinosa, Madrid)**

14:00 **Peter Christie (Houston)**
Biogenesis of the T-DNA transfer system of *Agrobacterium tumefaciens*

14:30 **Clarence Kado (Davis)**
Conservation of a C2H2-zinc finger-containing regulator of virulence and oncogenes of the Ti plasmid

15:00 **Stephen Farrand (Urbana)**
Ti plasmid conjugal donors: Turning them on, turning them off

15:30 **Coffee Break**

16:00 **Laura Frost (Edmonton)**
Repression and epidemic spread: Two levels of *traJ* regulation in the F plasmid

16:30 **Fernando de la Cruz (Santander)**
Bacterial conjugation: A two-step mechanism for DNA transport

17:00 **Trevor Lawley (Edmonton)**
Bacterial conjugative transfer: Visualization of successful mating pairs and plasmid establishment in live *Escherichia coli*

17:30- **Gunther Koraimann (Graz)**
17:50 Interaction of the relaxosomal protein $TraM_{R1}$ with the inner membrane protein $TraD_{R1}$: Coupling the DNA substrate to the transporter

19:00 **Dinner**

20:00 – 23:00 **Poster Session I (Replication; Partition/Stability)**

## *Tuesday June 25*

07:30 **Breakfast at the Union**

**TRANSFER II (Chairperson: Diane Taylor, Edmonton)**

08:30 **Keynote Address: Richard Meyer (Austin)**
Some new connections between replication and conjugal DNA transfer

09:10 **Steven Matson (Chapel Hill)**
The multiple roles of TraI in conjugative transfer of F plasmid

09:40 **Joel Schildbach (Baltimore)**
Mapping the active site of the F factor TraI relaxase activity

10:10 **Coffee Break**

10:40 **Beth Traxler (Seattle)**
Analysis of protein-protein interactions between the F plasmid conjugal DNA transfer proteins TraD, TraM, and TraI

11:00 **Manuel Espinosa (Madrid)**
Features of the pMV158-encoded protein MobM and the plasmid origin of transfer, *oriT*

11:30 **Jamie Caryl (Leeds)**
Initial events in small staphylococcal plasmid transfer

11:50 **Christopher D. Thomas (Leeds)**
Dimerization of the RepD initiator protein is a specific and stage-regulated process

12:10 **Dominique Galli (Indianapolis)**
A DNA invertase spoils conjugation for pVT745

12:30 **Lunch**

**TRANSFER III (Chairperson: Laura Frost, Edmonton)**

14:00 **Don Clewell (Ann Arbor)**
*Enterococcus faecalis* sex pheromone systems: Transfer origins of pAD1 and pAM373 and genetic analyses of the corresponding pheromones

14:30 **Gary Dunny (Minneapolis)**
Molecular and genetic dissection of the mechanism of negative control of pheromone-inducible conjugation in the *Enterococcus faecalis* pCF10 system

14:50 **Keith Weaver (Vermillion)**
Pursuing the target of the pAD1 *par* post-segregational killing system

15:10 **Coffee Break**

15:40 **Elisabeth Grohmann (Berlin)**
Conjugative transfer of broad-host-range plasmids in Gram-positive bacteria

**PLASMIDS and BIOFILMS (Chairperson: Don Clewell)**

16:00 **Jean-Marc Ghigo (Paris)**
Natural conjugative plasmids induce bacterial biofilm development

16:30- **Ellen Zechner (Graz)**
17:00 *Escherichia coli* biofilm architecture induced by derepressed IncF plasmids

19:00 **Dinner**

20:00 – 23:00 **Poster Session II (Partition/Stability; Transfer)**

## *Wednesday June 26*

07:30 **Breakfast at the Union**

**PLASMID EVOLUTION AND DIVERSITY (Chairperson: Patricia Sobecky, Atlanta)**

08:30 **Keynote Address: A. Mark Osborn (Colchester)**
(Conjugative) genomic islands as the fifth columnists of bacterial adaptation: Evolutionary lessons from the sequence analysis of R391

09:10 **Chris M. Thomas (Birmingham)**
Cooperative interactions in the regulation of replication, maintenance and transfer of IncP-1 plasmid

09:40 **Douglas Rawlings (Matieland)**
Interaction between two related IncQ-like plasmids isolated from bacteria that grow in the same environment

10:10 **Coffee Break**

10:40 **David Romero (Cuernavaca)**
Gene conversion leads to concerted evolution between members of a plasmidic multigene family in *Rhizobium*

11:10 **Darja Zgur-Bertok (Ljubljana)**
Colicin K synthesis is posttranscriptionally regulated by the stress alarmone ppGpp and is expressed in up to 3 % of the colicinogenic population

11:30 **Patricia Vary (DeKalb)**
Comparison of sequences of five homologous theta replicons from *B. megaterium* QM B1551 and progress in complete sequencing of the seven plasmid array

11:50 **Ichizo Kobayashi (Tokyo)**
Restriction modification systems as selfish mobile genetic elements maintaining and rearranging the genome

12:10 **Picnic Lunch (Barbeque) and Free Afternoon (informal activities)**

19:00 **Dinner**

20:00 – 23:00 **Poster Session III (Evolution/Diversity; Ecology, Virulence/Antibiotic Resistance, Pathogenicity Islands)**

## *Thursday June 27*

07:30 **Breakfast at the Union**

**PLASMID ECOLOGY (Chairperson: Douglas Rawlings, Matieland)**

08:30 **Keynote Address: Patricia Sobecky (Atlanta)**
Marine plasmids: Insights from diversity studies and sequencing

09:10 **Eva Top (Moscow, Idaho)**
IncP-1beta plasmids and chloaromatic degrading beta-proteobacteria, from alpha to omega

09:40 **Sarah Turner (Oxford)**
Determining the ecological mechanisms of plasmid adaptation to the niche in plant associated pseudomonad populations

10:10 **Coffee Break**

**VIRULENCE/ANTIBIOTIC RESISTANCE PLASMIDS (Chairperson: Richard Novick)**

10:40 **Keynote Address: Jorge Crosa (Portland)**
The pJM1 plasmid encodes iron transport and regulatory genes that are essential in microbial pathogenesis

11:20 **Julian Rood (Clayton)**
Functional analysis of the Tn*4451/4453* family of clostridial mobilisable transposons

11:50 **Malabi Venkatesan (Silver Spring)**
The virulence plasmid sequence of *Shigella flexneri*

12:20 **Manuela Di Lorenzo (Portland)**
Novel domain organization of AngM, a nonribosomal peptide synthetase essential for siderophore biosynthesis in *Vibrio anguillarum*

12:40 **Lunch**

**PATHOGENICITY ISLANDS/GENE TRANSFER ELEMENTS (Chairperson: Dhrupad Chattooga)**

14:00 **Keynote Address: Richard Novick (New York)**
Mobile genetic elements encoding toxinosis-causing bacterial toxins: the superantigen-encoding pathogenicity islands of *S. aureus*

14:40 **Graham Hatfull (Pittsburgh)**
Comparative bacteriophage genomics

15:10 **Jeffrey Lawrence (Pittsburgh)**
Constraints on lateral gene transfer in prokaryotes

15:40 **John Beaber (Boston)**
Environmental and genetic control of the SXT, a conjugative antibiotic resistance gene transfer element from *Vibrio cholerae*

16:00 **Coffee Break**

**PLASMIDS AS TOOLS (Chairperson: David Romero, Cuernavaca)**

16:30 **Joseph Sturino (Raleigh)**
Antisense RNA based bacteriophage defense strategies

17:00 **Jadwiga Wild (Madison)**
The copy-control expression vectors (pBAC/oriV/TrfA) with the tightly controlled *ara* and *rha* promoters

17:20- **Richard Fekete (Bethesda)**
17:40 The role of IHF in P1 plasmid replication

**19:30 – 23:00 Banquet**

## *Friday June 28*

07:30 **Breakfast at the Union**

**RAPPORTEURS (Chair: Clarence Kado, Davis)**

09:00 **Anne Summers (Athens, Georgia)**
Let's sequence the floating genome!

09:30 **Tatiana Tatusova (NCBI, NLM, NIH)**
Complete genome resources at NCBI

10:00 **Society for Plasmid Biology**

10:30 **Christopher M. Thomas (Birmingham)**
SUMMARY AND HIGHLIGHTS

11:30 **Lunch**

12:00 **Departure**

# *REPLICATION*

## ORAL PRESENTATIONS

**12** Oligomerization of the yeast replication terminator protein, Fob1p is critical for its ability to arrest replication forks. Deepak Bastia, Bidyut K. Mohanty and Arabinda Das

**13** Host specific mechanisms for helicase complex formation at the origin of a broad host range plasmid. Igor Konieczny, Marcin Pacek, Yong Jiang, Donald R. Helinski and Aresa Toukdarian

**14** Monomers and dimers of π Protein and what they do to make plasmid R6K happy. Marcin Filutowicz, Ricardo Kruger, Andrea Dudding, Sheryl Rakowski, Selvi Kunnimalaiyaan and Jianwei Wu

**15** Relaxation of autorepression of the initiator gene of plasmid P1 relaxes the plasmid copy number control. Nilangshu Das, Majda Valjavec-Gratian, Richard Fekete and Dhruba K. Chattoraj

**16** A specific region in the N-terminus of the TrfA-44 replication initiation protein of plasmid RK2 is required for recruitment of *Pseudomonas aeruginosa* DnaB helicase to the plasmid origin. Zhenping Zhong, Donald Helinski, and Aresa Toukdarian

**17** Structural changes in the pPS10 replication initiator, RepA, upon binding to origin DNA. Teresa Díaz-López, Marta Lages-Gonzalo, Ana Serrano-López, Carlos Alfonso, Germán Rivas, Ramón Díaz-Orejas and Rafael Giraldo

**18** Antisense RNA in plasmids and beyond. E. Gerhart H. Wagner

**19** Antisense RNA-mediated transcriptional attenuation: A comparison between Gram-positive bacteria and *E. coli*. Sabine Brantl and E. Gerhart H. Wagner

**21** Replication of the linear plasmid LP17 from *Borrelia burgdorferi*, the Lyme disease spirochete. George Chaconas, Kerri Kobryn, Yvonne Tourand, and Cecile Beaurepaire

**22** Role of the PcrA helicase in the replication of rolling-circle plasmids. Saleem A. Khan, Tseh-Ling Chang, Asma Naqvi, Gabriela Kramer, Rajan Munshi and Syam P. Anand

**24** Identification of a novel gene product that suppresses the toxicity of a TrfA peptide from plasmid RK2 and its relationship to the DnaA host initiation protein. Trevor Banack, Peter D. Kim, and William Firshein

26 Replication of lambda plasmids carried out by the heritable replication complex. Sylwia Baranska, Katarzyna Potrykus, Grazyna Konopa, Alicja Wegrzyn, Grzegorz Wegrzyn

27 Plasmids of the ColE2 family with initiator proteins that synthesize the origin-specific primers. T. Itoh, M. Yagura, S. Nishio, M. Han, T. Nagase, K. Ogiwara, T. Tanaka and K. Aoki

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30 Comparative characterization of *repABC* type replicons of *Paracoccus pantotrophus* composite plasmids. Dariusz Bartosik, Jadwiga Baj, Ewa Piechucka, Edyta Zbiciak and Miroslawa Włodarczyk

33 CopR binds and bends its target DNA: structure and sequence of the DNA in the CopR-DNA complex. Katrin Steinmetzer, Joachim Behlke, Mike Lorenz, Peggy Freede and Sabine Brantl

35 Complete nucleotide sequence and characterization of replication and stable maintenance functions of a highly disseminating plasmid encoding the CTX-M-3 β-lactamase. Marcin Golebiewski, Anna Kulinska, Jolanta Mierzejewska, Izabella Kern-Zdanowicz, Grazyna Jagura-Burdzy and Piotr Ceglowski

37 Interactions between the pMV158-encoded RepB protein and its double-stranded origin of replication. José A. Ruiz-Masó, Manuel Espinosa, and Gloria del Solar

39 Interactions of the transcriptional repressor CopG with its target DNA. Ana M. Hernández-Arriaga, Manuel Espinosa, and Gloria del Solar

40 Roles of dimerization surfaces of an initiator protein in replication control. Ricardo Krüger, Jianwei Wu and Marcin Filutowicz

41 π protein-dependent and π-independent transitions from "closed" to "open" complexes at the γ ori of plasmid R6K. Ricardo Krüger and Marcin Filutowicz

42 Dimeric isomers of replication initiator: DNA binding activities and regulatory roles in replication and transcription. Ricardo Krüger, Sara Snyder, Selvi Kunnimalaiyaan, Ross B. Inman and Marcin Filutowicz

43 Antagonistic binding effect of oligomers of a replication initiator protein (π) to the direct repeats in plasmid R6Kγ ORI. Ricardo Krüger and Marcin Filutowicz

44 A promoter for *oop* RNA synthesis has a role in replication of plasmids derived from bacteriophage lambda. Katarzyna Potrykus, Ewa Perzylo, Grzegorz Wegrzyn

Oligomerization of the yeast replication terminator protein, Fob1p is critical for its ability to arrest replication forks

Deepak Bastia, Bidyut K. Mohanty and Arabinda Das. Department of Biochemistry and Molecular Biology, MUSC, Charleston, SC 29425

We have dissected the functional domains of *S. cerevisiae* replication terminator protein Fob1p by site-directed mutagenesis and have purified the protein to study its biochemistry with the long-term goal of unraveling the mechanism of replication fork arrest in yeast rDNA. Purified Fob1p was expressed in *E. coli* and purified and its DNA binding behavior was analyzed. Unlike bacterial replication terminator proteins, Fob1p is not a sequence-specific DNA binder but appears to have general DNA binding activity. The results imply that Fob1p probably interacts with 2 or more sequence-specific DNA binding proteins that load Fob1p at the two tandem replication termini located in the nontranscribed spacer region of rDNA repeats on chromosome 12.

Extensive mutagenesis using the Ade2 +/- sectoring colony color phenotype yielded several mutants of the gene that were partially or completely defective in replication fork arrest. These mutant forms of the protein and the wild type protein were analyzed by GFP tagging and were localized to the yeast nucleus. Some of the mutant forms were proficient in binding to YDR026C protein that by monohybrid analysis appeared to bind to the terminus region. Thus at least some of the mutant forms did not have a global defect in protein misfolding. Yeast 2-hybrid analysis showed that wild type Fob1p interacted with itself whereas the mutant forms defective in replication termination were deficient in self-interaction and presumed to produce monomeric proteins. From the analyses presented above, we conclude that oligomerization of Fob1p is critical for its ability to stop replication forks.

Host specific mechanisms for helicase complex formation at the origin of a broad host range plasmid.

Igor Konieczny[1], Marcin Pacek[1], Yong Jiang[2], Donald R. Helinski[2] and Aresa Toukdarian[2]

[1]Department of Molecular and Cellular Biology, Faculty of Biotechnology, University of Gdansk, Kladki 24, 80-822 Gdansk, Poland, [2]Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, CA 92093-0322, USA

The structure of a replication origin (*ori*) and its interaction with replication initiation proteins determine the replicon specific mechanism for DNA replication initiation. Generally, binding of replication initiation proteins (Rep) at specific iterated nucleotide sequences cause local unwinding of an A+T-rich region in *ori*, which provides the entry site for helicase. In addition to unwinding double-stranded DNA the helicase has to be correctly positioned within the opened region on to the separated DNA strands. The molecular mechanism(s) of that event is not fully understood. An outstanding question pertaining to DNA replication of broad-host-plasmids is the mechanism or mechanisms of recruitment and loading of host helicase during replication initiation in diverse bacterial species.

We have found that depending upon the host bacterium, the broad-host-range plasmid RK2 utilizes different pathways for helicase recruitment and activation. The first pathway recruits bacterial helicase *via* interaction with the host DnaA replication protein bound at four sequences (DnaA-boxes) located in the plasmid replication origin (*oriV*). This pathway is responsible for RK2 replication initiation in *Escherichia coli* but not in *Pseudomonas aeruginosa*. The second pathway relies upon helicase recruitment through five direct repeat sequences (iterons) in *oriV* bound by the longer (44 kDa) version of the plasmid replication initiation protein TrfA and is responsible for RK2 replication in *Pseudomonas sp.* but not in *E. coli*. Moreover, the host replication protein DnaA is not essential for the *Pseudomonas sp.* helicases complex formation and activity at *oriV* in the presence of TrfA-44. The short (33 kDa) version of TrfA does not support helicase complex formation in the absence of the DnaA protein.

Our results bring a new understanding of the strategy for initiation of DNA replication of a broad-host-range replicon in different bacterial species. It is now evident that the structure of the origin combined with two versions of a plasmid replication initiation protein provides alternative pathways for host helicase recruitment and activation by plasmid RK2 depending on the bacterial host.

MONOMERS AND DIMERS OF □ PROTEIN AND WHAT THEY DO TO MAKE PLASMID R6K HAPPY

Marcin Filutowicz, Ricardo Kruger, Andrea Dudding, Sheryl Rakowski, Selvi Kunnimalaiyaan and Jianwei Wu

Department of Bacteriology, University of Wisconsin, Madison, WI 53706, USA

In biological systems, intracellular DNA is organized into functional units called replicons. Typical replicons such as chromosomes or plasmids are stably maintained at a constant copy number within the cell. For this to occur, DNA synthesis must be sensitive to the concentration of replicons, utilizing regulatory circuits to boost low copy numbers and reduce elevated ones. Precise regulation of copy control is achieved in one major class of bacterial plasmids by interactions between an essential, autoregulated, plasmid-encoded replication protein (Rep) and iterated binding sites (iterons) in the origin of replication (*ori*).Two models, not mutually exclusive, were proposed to explain the regulatory roles of iteron-Rep interactions. In the first model, sets of iterons compete for Rep protein. In the second model, Rep-iteron associations facilitate a "handcuffed" complex that reduces the capacity for replication. Additionally, in certain plasmids, the autoregulatory circuit involves the binding of replication protein to a particular inverted repeat (IR), separate from the set of iterons in the origin. We will present the □ *ori* replicon of *Escherichia coli's* plasmid R6K and discuss existing observations that support and those that argue against each of the aforementioned models.

# RELAXATION OF AUTOREPRESSION OF THE INITIATOR GENE OF PLASMID P1 RELAXES THE PLASMID COPY NUMBER CONTROL

Nilangshu Das, Majda Valjavec-Gratian, Richard Fekete and Dhruba K. Chattoraj

Lab of Biochemistry, The Center for Cancer Research, NCI, NIH, Bethesda, MD 20892-4255

P1 plasmid replication initiator protein RepA binds to the plasmid origin at multiple sites, called iterons. Nested within the iterons lies the promoter for the *repA* gene. RepA binding to the origin iterons allows initiation as well as repression of the *repA* promoter. This autorepression is >99% efficient but requires proper folding of RepA by chaperone proteins (DnaJ,K,GrpE). Previously, mutants of RepA were isolated that were efficient in autorepression without requiring the chaperones (1). The mutants were initiation proficient and increased the plasmid copy number (the copy-up phenotype) in 10/12 cases. The results suggest that the two phenotypes of RepA, chaperone-requirement for iteron binding and control of plasmid copy number, have a common basis.

To explore the correlation of the two phenotypes further, we took an opposite approach. We selected new copy-up mutants of RepA and asked if they have become independent of chaperones for iteron binding and replication. In 8/12 mutants, the two phenotypes were not correlated- the mutants still required chaperones for efficient autorepression and replication. Thus relaxing copy number control appears necessary but not sufficient for RepA folding.

It is currently believed that properly folded RepAs have a reduced dimerization efficiency, and the plasmid copy number control operates by allowing interactions between iteron-bound RepA monomers (handcuffing) (2). Our results would therefore suggest that the domains involved in dimerization and in handcuffing may overlap but they are not identical.

Three of the 12 newly isolated copy-up mutants were defective in iteron binding, and required higher level of proteins for autorepression. Apparently, the *repA* promoter was on longer till the protein level increased enough to affect autorepression. However, as in the chaperone-independent mutants, the copy-up phenotype was unexpected. The results suggest that mechanisms of autorepression and plasmid copy number control have a common basis.

The results are consistent with our earlier suggestion that handcuffing helps to maintain autorepression (3). To understand the relationship of dimerization, chaperone requirement, autorepression and handcuffing to copy number control, we are characterizing the three autorepression defective mutants in vitro.

1). Sozhamannan & Chattoraj (1993) J. Bact. 175, 3546-55. 2). Park et al (2001) EMBO J 20, 7323-32. 3). Chattoraj et al (1988) Cell 52, 551-7.

**A SPECIFIC REGION IN THE N-TERMINUS OF THE TrfA-44 REPLICATION INITIATION PROTEIN OF PLASMID RK2 IS REQUIRED FOR RECRUITMENT OF *PSEUDOMONAS AERUGINOSA* DnaB HELICASE TO THE PLASMID ORIGIN**

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The broad-host-range plasmid RK2 encodes two versions of the essential replication initiation protein, TrfA, by using two in-frame translation starts spaced 97 amino acids apart. The smaller initiation protein, TrfA-33, is sufficient for plasmid replication in many bacteria. Efficient plasmid replication in *Pseudomonas aeruginosa* however requires the larger TrfA-44 protein. Recently it was shown that TrfA-44 alone could recruit and activate the DnaB helicase of *P. aeruginosa* or *P. putida* on to the RK2 origin in vitro (see abstract by Konieczny, I. et al). By contrast, the TrfA-33 protein requires *P. putida* DnaA protein to load and activate the helicase of *P. putida* in vitro and requires the *E. coli* DnaA plus DnaC proteins to load the helicase of *E. coli*. These results suggested a role for the N-terminal 97 amino acids of TrfA-44 in helicase loading in *Pseudomonas*.

With the aim of identifying amino acids residues required for stable RK2 replication in *P. aeruginosa*, specific deletions within the N-terminal 97 amino acids unique to TrfA-44 were constructed. The in vitro activity of purified mutant proteins in recruiting and loading helicase was tested as was the ability of the mutant proteins to support mini-RK2 replication in *P. aeruginosa* in vivo. Deletion of TrfA-44 amino acids 21-32 resulted in a mutant protein, TrfA-44Δ2, that had lost the ability to bind and load the DnaB helicase of *P. aeruginosa* or *P. putida* on to the RK2 origin in vitro. This mutant, however, was fully functional at the RK2 origin in recruiting and activating the *E. coli* DnaB helicase in the presence of *E. coli* DnaC and DnaA proteins. The TrfA-44Δ2 protein was also defective in supporting stable replication of an RK2 mini-replicon in vivo in *P. aeruginosa*. The deletion of amino acids 37-55 (mutant TrfA-44Δ3) or a point mutant (mutant TrfA-44E22A) resulted in only a slight reduction in activity in vitro with the *Pseudomonas* helicases, while deletion of amino acids 71-88 (TrfA-44Δ4) had no effect on either in vitro or in vivo activities of the mutant TrfA-44 protein.

The interaction of DnaB with a plasmid encoded replication initiation protein is not unique to RK2. The replication initiation proteins of plasmids R6K (Pi protein) and pSC101 (RepA) as well as the chromosomal initiator protein DnaA have been shown to interact with *E. coli* DnaB. However, the role of TrfA-44 as being solely responsible for helicase loading and activation is exceptional amongst plasmid replicons.

## STRUCTURAL CHANGES IN THE pPS10 REPLICATION INITIATOR, RepA, UPON BINDING TO ORIGIN DNA

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RepA protein is the DNA replication initiator of the *Pseudomonas* plasmid pPS10 (1). RepA dimers bind to an inversely-repeated operator sequence, thus repressing its own synthesis (2), whereas monomers bind to four directly-repeated sequences (iterons) to initiate DNA replication (3). We had previously proposed that RepA is composed of two "Winged-Helix" (WH) domains (4), a structural unit also present in eukaryotic and archaeal initiators (5). In order to bind to the whole iteron sequence through both WH domains (4,6), RepA should couple monomerization to a conformational change in the N-terminal WH, which includes a Leucine Zipper-like motif (LZ) involved in dimerization (7,3). We will show that, by itself, binding to iteron DNA *in vitro*: i) dissociates RepA dimers into monomers and ii) alters RepA conformation, suggesting an allosteric effect. Furthermore, we also show that similar changes in RepA are promoted by mutations that substitute two Leu residues of the LZ by Ala, destabilizing the hydrophobic core of the first WH domain. We propose that this mutant (RepA-2L2A) would resemble a transient folding intermediate in the pathway leading to active monomers. These findings, together with the known activation of other Rep-type proteins by chaperones (8-11), are relevant to understand the molecular basis of plasmid DNA replication initiation.

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Antisense RNA in Plasmids and Beyond

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In 1981, antisense RNA control was discovered in two bacterial plasmids, ColE1 and R1. In both cases, these small, untranslated RNAs were shown to be the key regulators of replication and, thus, the copy number of the plasmids. Subsequently, many more examples of antisense RNAs were found, the vast majority of which reside in accessory genetic elements. Probably more than 100 antisense RNAs are plasmid-encoded and control replication frequency (by far the majority), conjugation and post-segregational killing. Some others regulate lysis/ lysogeny decisions in phage, and yet some more control transposition of insertion elements. There are a number of different mechanisms by which control is exerted, from block of translation, facilitated target RNA decay, induced transcriptional termination, and others. Plasmids have been the model systems of choice to illuminate the molecular details of antisense/ target RNA systems. I will review some of the current knowledge about the system under study in my group, the CopA/CopT system of plasmid R1.

Until recently it appeared as if antisense RNA control was almost exclusively confined to accessory elements. Bacterial chromosomes were known to encode at best a handful of antisense RNAs, such as OxyS, DsrA, MicF, all of which are stress response regulators. In addition to their chromosomal location, these three have another distinguishing feature when compared to the plasmid-borne ones. They are *trans*-encoded. I.e. antisense and target genes are not co-located, and thus complementarity between the two RNAs is non-contiguous. In contrast, almost all antisense RNAs in plasmids are *cis*-encoded. Here, antisense and target RNA are transcribed off the same segment of DNA (in opposite orientation), and complementarity therefore is complete.

In this talk I will argue that the very rare occurrence of chromosomally encoded antisense RNAs is a "historical artifact". Based on recent results, it is clear that *Escherichia coli* carries many genes encoding small, untranslated RNAs [1]. It is likely that many of them, probably the majority, are antisense RNAs in search of a biological role [4].

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## ANTISENSE-RNA-MEDIATED TRANSCRIPTIONAL ATTENUATION: A COMPARISON BETWEEN GRAMPOSITIVE BACTERIA AND *E. COLI*

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Antisense RNA-mediated transcriptional attenuation is a regulatory mechanism operating in replication control of two groups of plasmids in Gram-positive bacteria, the pT181 group and the *inc18* family, represented by pIP501. In this control mechanism, the fate of the target (sense) RNA is affected by the antisense RNA. Upon binding of antisense-RNA, the nascent transcript folds into one of two mutually exclusive conformations, so that a Rho-independent terminator forms, entailing premature termination. If the antisense RNA fails to bind during a critical time interval of transcription, the nascent RNA refolds into an alternative structure which prevents termination and promotes read-through. Details of this type of control were characterized *in vivo* for pIP501 and later on *in vitro* for both pIP501 and pT181. A comparison of the *in vitro* data for both plasmids revealed similarities but also several differences. In contrast to Gram-positive bacteria, this control mechanism has so far not been reported in Gram-negative bacteria or their plasmids.

In the work presented here we asked whether such a mechanism can be supported by *Escherichia coli*. The core replication control regions of plasmids pT181 and pIP501, respectively, were transferred into this heterologous host. Using *in vivo lacZ* reporter gene assays, it was shown that the antisense RNAs of these plasmids can inhibit *lacZ* expression, and that most of this effect can be accounted for by reduced mRNA read-through. Northern analyses confirmed that the ratio of attenuated over read-through target RNA was increased in the presence of the cognate antisense RNA, as expected for this mechanism. Similarly, both antisense RNAs induced premature termination of their cognate target RNAs in an *E. coli in vitro* transcription system, whereas the noncognate antisense RNAs had no effect. Thus, this report shows that antisense RNA –mediated transcriptional attenuation is supported by at least one Gram-negative host, although the data indicate that inhibitory efficiencies are lower than in, e.g., *Bacillus subtilis*. Possible explanations for the apparent absence of this control mode in plasmids of Gram-negative bacteria are discussed.

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REPLICATION OF THE LINEAR PLASMID LP17 FROM BORRELIA BURGDORFERI, THE LYME DISEASE SPIROCHETE

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*Borrelia burgdorferi,* a causative agent of Lyme disease, has a unique segmented genome containing a linear chromosome and about 12 linear and nine circular plasmids (Casjens *et al.*, 2000). The linear replicons have covalently closed hairpin ends referred to as "telomeres" (Casjens, 1999). Replication of these DNA molecules is believed to involve bidirectional replication from the center of the linear molecule (Picardeau *et al.*, 1999) to generate a circular head-to-head, tail-to-tail dimer (see Kobryn & Chaconas, 2001). The dimer junctions are subsequently processed in a DNA breakage and reunion reaction referred to as telomere resolution (Chaconas *et al.*, 2001). The telomere resolvase, ResT, has recently been purified and is related in mechanism to the tyrosine recombinases and type 1B topoisomerases (Kobryn & Chaconas, 2002).

Studies are currently underway to define the regions required for replication of the *B. burgdorferi* 17 kb linear plasmid, lp17. We are also defining the requirements for a functional *B. burgdorferi* telomere and further characterizing the mechanism of action of ResT.

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**Role of the PcrA Helicase in the Replication of Rolling-Circle Plasmids**

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A large number of small, multicopy plasmids in Gram-positive bacteria replicate by a rolling circle (RC) mechanism (reviewed in ref. 1). Plasmids of the pT181 family in *Staphylococcus aureus* encode an initiator (Rep) protein that functions as a dimer during RC replication (2-5). Previous studies have identified the DNA binding and nicking domains of the RepC protein encoded by the pT181 plasmid (6, 7). Mutational analyses have also shown that the DNA binding and nicking activities of RepC can be uncoupled, and both of these activities are required for replication (7). Initiation of replication of RC plasmids involves sequence-specific binding of the Rep protein to the origin of replication (8). The Rep protein subsequently nicks one strand of the DNA within the origin (9). The 3' OH end at the nick site then serves as a primer for displacement synthesis which presumably involves unwinding of the DNA by the PcrA helicase ahead of the replication fork (10). During the termination of plasmid RC replication, the initiator protein covalently attached to the DNA is expected to catalyze additional transesterification reactions leading to the release of the parental circular leading strand of the DNA and a SC DNA containing a newly synthesized leading strand. These events require that the 5' end of the displaced DNA strand bound to the initiator be present in close proximity to the replication fork as it approaches the termination site, i.e. the regenerated origin sequence. It is generally assumed that an interaction between a replisome protein and the plasmid initiator protein may facilitate this juxtaposition.

The *S. aureus* PcrA helicase was identified several years ago and found to be required for the RC replication of plasmid pT181 (10). Genetic studies in *S. aureus* and *Bacillus subtilis* have shown that this helicase is required for cell viability, and may also play a role in UV repair and replication of the chromosomal DNA (10, 11). The *pcrA* gene has also been identified in several other Gram-positive bacteria such as *Bacillus stearothermophilus, Lactococcus lactis*, *Streptococcus pyogenes* and *Streptomyces coelicolor*, and it is likely that they have similar functions in their respective hosts. The PcrA helicase of *B. stearothermophilus* has been purified and its crystal structure determined (12). It has a 3' → 5' helicase activity, a limited 5' → 3' activity, and it acts as a monomer in contrast to several other replicative helicases which act as hexamers (13) The *B. stearothermophilus* PcrA helicase activity is not very processive, but the RepD initiator protein encoded by the *S. aureus* plasmid pC221 enhances its processivity (14). We have studied the role of the *Staphylococcus aureus* PcrA helicase in plasmid pT181 replication. Purified PcrA helicase supported the *in vitro* replication of plasmid pT181 when added to cell extracts prepared from a *pcrA* mutant strain that is defective in pT181 replication. These results showed a direct requirement of PcrA helicase in pT181 RC replication. PcrA was found to have a robust ATPase activity that was stimulated in the presence of DNA. Unlike many replicative helicases, PcrA was highly active as a 5' →3' helicase, and had a weaker 3 →5 helicase activity. PcrA was also able to efficiently unwind ds oligonucleotides containing 4 nt long 5' ss tails. We also report that PcrA is able to unwind supercoiled (SC) pT181 DNA nicked at the origin by the RepC initiator

protein. The unwinding of the nicked DNA by PcrA required both sequence-specific noncovalent binding of RepC to the origin, as well as the presence of covalently attached RepC at the nick site. These data suggest that PcrA is recruited to the nick site through its interaction with RepC and subsequently initiates DNA unwinding at the origin. Using *in vitro* pull-down assays, we also demonstrate a direct protein-protein interaction between RepC and PcrA. Our data support a model in which PcrA is recruited to the pT181 origin through its interaction with RepC. PcrA unwinds the DNA ahead of the replication fork and the protein-protein interaction between RepC and PcrA results in the localization of the RepC-bound 5' end of the displaced pT181 DNA strand near the moving replisome. When the replication fork reaches the termination site, i.e. the regenerated origin sequence, RepC attached to the displaced 5' end of the leading strand is appropriately positioned to catalyze DNA cleavage-rejoining events, resulting in the release of ss, circular leading strand of the DNA.

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# IDENTIFICATION OF A NOVEL GENE PRODUCT THAT SUPPRESSES THE TOXICITY OF A TRFA PEPTIDE FROM PLASMID RK2 AND ITS RELATIONSHIP TO THE DNAA HOST INITIATION PROTEIN

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The broad host range plasmid RK2 is capable of replication and stable maintenance within a wide range of Gram negative bacterial hosts. Carrying resistance genes to three antibiotics (amp, Kan, Tet) it has been isolated from a number of different medical environments. Despite the diversity of host cells, only two plasmid encoded loci are necessary to initiate unidirectional replication, the cis acting origin of replication (oriV) and the transacting initiator gene trfA which codes for two proteins 44kDa and 33 kDa, the latter of which is expressed from an internal translational start within TrfA (Smith and Thomas, 1984). Recently, specific binding of TrfA and the host initiation protein of Escherichia coli DnaA to oriV were found to be required for initiation of plasmid replication, consistent with the presence of four DnaA boxes within the oriV sequence (Konieczny et al, 1997). It appears that the DnaA protein cannot by itself form an open complex in oriV but enhances its formation by TrfA. Nevertheless, numerous experiments have failed to demonstrate a direct physical interaction between TrfA and the DnaA protein although experiments using TrfA and the ClpX chaperone protein did suggest a physical interaction (Konieczny and Zylicz, unpublished results).

Another important aspect of RK2 replication is that it has been associated with the inner membrane of a number of Gram negative hosts (Banack et al., 2000). The TrfA initiation proteins were detected in both inner and outer membrane fractions of their hosts (Michaels et al., 1994, Banack and Firshein 2000) but further studies in E. coli revealed that oriV, the TrfA initiator proteins, and replication inhibited by specific anti TrfA antibody were enriched in a specific subdomain derived from the inner membrane fraction representing less than 10% of the total membrane (Mei et al.,1995; Kim and Firshein, 2000). Finally, although not mentioned specifically, Pogliano et al (2001) have localized RK2 to clusters in their E. coli host that are targeted to specific locations which could involve membrane receptors (Helinski, personal communication).

Numerous parallel studies with E. coli have also suggested that in vivo replication is membrane associated. The DnaA protein is located at the cell Membrane (Newman and Crooke, 2000), is activated by anionic phospholipids (Sekimizu and Kornberg, 1988), and the origin of replication (oriC) has been found to bind to the same inner membrane subdomain as oriV (Chakraborti et al, 1992).

Previous studies in our laboratory have examined the structure of the TrfA proteins of RK2 in relation to its function (Kim et al, 2000). Two membrane binding domains were detected, one of which was related to its ability to function as an initiation protein, while the other was part of a peptide that exhibited a severe toxicity for host cells. One possible explanation for this toxicity was the result of an interaction between the peptide and some necessary host protein, and that, if this were the case, it should be possible to overcome the toxicity by transformation with a vector containing additional

copies of the unknown host protein. An E. coli library constructed by D. Oliver was used to select for host sequences, which when placed in trans of the toxic TrfA peptide, would be capable of rescuing the host cells.

Using the strategy outlined above, a single open reading frame (ORF) that codes for a protein possessing extensive homology with the DnaA protein (but half its size), a DnaA paralog (Dp) was isolated. It is identical to a gene that codes for a protein (Hda) recently reported by Kato and Katayama (2001) that may help regulate the E. coli replication cycle and is also related to a partially purified factor (The IdaB protein) reported earlier by Katayama et al (1998) that is involved with DNA polymerase III in inactivating of the DnaA protein after initiation.

Using a tagged version of this protein, it was demonstrated that the paralog interacts physically with TrfA in vitro (as determined by immuno-affinity assays) unlike the DnaA protein and is enriched in membrane fractions in a manner similar to that of the TrfA and DnaA proteins. Additional experiments with a knockout strain of the paralog (constructed by E. Crooke) demonstrated a stimulation of plasmid transformation but the effects were not consistent. Further analysis of binding of the paralog to DnaA boxes within OriV as well as its effects on replication of membrane associated RK2 replication will be presented.

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# REPLICATION OF LAMBDA PLASMIDS CARRIED OUT BY THE HERITABLE REPLICATION COMPLEX

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There are two 'pathways' of replication of plasmids derived from bacteriophage lambda (lambda plasmids) in *Escherichia coli*. One pathway requires the assembly of a new replication complex before replication and the second pathway is based on the activity of the replication complex inherited by one of two daughter plasmid copies after a preceding replication round. Such a phenomenon was postulated to occur also in other replicons, including *Saccharomyces cerevisiae* ARSes.

We investigated directionality of lambda plasmid replication carried out by the heritable and newly assembled replication complexes. Using two dimensional agarose gel electrophoresis and electron microscopy we demonstrated that both in normal growth conditions and during the relaxed response to amino acid-starvation (when only replication carried out by the heritable complex is possible), bidirectionally and undirectionally replicating plasmid molecules occurred in host cells in roughly equal proportions. These results are compatible with the hypothesis that both complexes (heritable and newly assembled) are equivalent.

Apart from some suggestions based on genetic experiments, composition of the lambda heritable replication complex remained unknown. Using a procedure based on *in vivo* cross-linking, cell lysis, immunoprecipitation with specific sera, de-cross-linking and PCR analysis, we demonstrate that the lambda heritable replication complex consists of O, P, DnaB and, perhaps surprisingly, DnaK proteins.

## PLASMIDS OF THE COLE2 FAMILY WITH INITIATOR PROTEINS THAT SYNTHESIZE THE ORIGIN-SPECIFIC PRIMERS.

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The 17 classic ColE2 (ColE2-P9)-related plasmids are unique among many other plasmids in that their initiator proteins (Rep proteins) are origin-specific primases. The ColE2-P9 Rep synthesizes a primer RNA (ppApGpA) at the ColE2-P9 origin, which is specific for initiation of DNA replication by the host DNA polymerase I. The Rep protein consists of at least three functional regions (domains) responsible for the sequence-specific binding to the origin, primer RNA synthesis and possible specific interaction with DNA polymerase I.

The ColE2-P9 origin region is divided into two subregions: one involved in specific recognition and binding by the Rep protein and the other involved in the primer RNA synthesis followed by initiation of DNA replication. The origin region may be further divided into three functional regions (domains): the first one important for stable binding of the Rep protein but apparently not for replication, the second one important for binding and for replication (it contains the boundary of the two subregions) and the third one important for replication but apparently not for binding.

Expression of the ColE2-P9 Rep protein is regulated by a small antisense RNA (RNA I) at a post-transcriptional level. RNA I is entirely complementary to the 5' nontranslated region of the Rep mRNA. Interaction of the Rep mRNA with RNA I inhibits translation of the Rep protein, although RNA I does not directly cover the ribosome binding site and initiation codon.

Sequence homology at the nucleotide level of the replicon regions as well as at the amino acid level of the Rep proteins of the 17 classic ColE2-related plasmids indicates that they all share common mechanisms for initiation of DNA replication and its regulation. Among them there are 5 (IncA) specificity groups for the RNAI antisense activity and 4 (IncB) specificity groups for the interaction of the Rep protein and origin. There are 9 combinations of the IncA and IncB types. These two systems seem to have evolved independently. Comparison of the nucleotide sequences of the 11 ColE2-related plasmids revealed mosaic structures in the basic replicons of these plasmids and showed that the basic replicons of these plasmids further diverged by exchanging subregions of the basic replicons. Homologous recombination and site-specific recombination seem to have been involved in such exchanges.

Plasmid ColE6 (ColE6-CT14 and ColE6-Ind8) has been known to be longer by 4 kb than other ColE2-related plasmids and to carry a second replicon. We determined the complete nucleotide sequences of ColE2-P9 and ColE6-CT14 plasmids. In plasmid ColE2-P9 we found a new type of the mobilization (mob) region downstream of the origin. In ColE6-CT14 homologous mob regions are repeated twice. Between the two regions there is a replicon of the narrow host range with the iterons and a DnaA box at the origin. Homologous recombination within the mob regions and/or recombination after nicking events at the transfer origins might have created a composite plasmid ColE6-CT14.

Recently a large number of new ColE2-related plasmids (including those encoding ColE2, ColE6 and ColE7) were isolated in Australia. Comparison of the nucleotide sequences of the ColE2 gene clusters of 9 new ColE2 plasmids and 6 classic ColE2 plasmids revealed two types of ColE2 gene clusters (Tan and Riley, 1997). It was suggested that the type II gene clusters (carried by the 9 new ColE2 plasmids and one classic ColE2 plasmid, plasmid ColE2-CA42) were generated by recombination between the type I ColE2 gene clusters (carried by the remaining 5 classic ColE2 plasmids) and the ColE7 gene cluster. We analyzed the replicon regions of the 9 new ColE2 plasmids and found that there are at least two replicon types among them. Four of them share a common replicon that is identical to that of plasmid ColE2-GEI602 with the type I ColE2 gene cluster. Three of the remaining 5 plasmids probably carry ColE1 type replicons and this seems to be a new combination of the ColE2 gene clusters and the replicon types.

**RepC-PcrA Interactions During the Rolling Circle Replication of Plasmid pT181**

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Members of the plasmid pT181 family found in Gram-positive bacteria replicate by a rolling-circle mechanism (1). The RepC initiator protein encoded by pT181, which acts as a dimer, nicks at the origin of replication and becomes covalently attached to the 5' end of the DNA. The 3' OH end at the nick site then serves as a primer for displacement synthesis which involves unwinding of the DNA by the PcrA helicase. An *S. aureus* mutant carrying the *pcrA3* mutation was shown to be defective in the RC replication of plasmid pT181, but this mutation did not effect chromosome replication or cell survival (2). Mutants in the RepC initiator protein of pT181 have been isolated that allow plasmid replication in the *pcrA3* strain, suggesting an interaction between PcrA and RepC proteins (3). We have purified the *S. aureus* PcrA helicase as a His-6 fusion and studied its biochemical properties. PcrA was highly active as an ATPase and its activity was further stimulated by ssDNA as well as supercoiled pT181 DNA nicked by the RepC protein. PcrA helicase had a robust 5'→3' helicase activity and a weaker 3'→5' activity. Incubation of pT181*cop608* DNA with RepC generates relaxed, covalently closed circular (CCC) DNA as well as nicked OC DNA. When pT181*cop608* DNA was incubated with both PcrA and RepC, a faster migrating band corresponding to the unwound "U" form of the DNA was observed. These data suggested that PcrA has origin-specific unwinding activity in the presence of a RepC generated nick. Two RepC mutants, nick$^+$ bind$^-$ and nick$^-$ bind$^+$ were also used in these experiments. Incubation of the DNA binding mutant of RepC (nick$^+$ bind$^-$) with SC pT181 DNA generated relaxed CCC DNA since this mutant has nicking-closing activities. However, addition of PcrA helicase to the reaction in the presence of this mutant did not generate any unwound U form of the DNA as observed with wild-type RepC. Incubation of PcrA with pT181 DNA in the presence of the nick$^-$ bind$^+$ RepC mutant, which is able to bind noncovalently to the pT181 origin but is defective in nicking-closing, did not change the migration pattern of SC pT181 DNA. These results showed that both DNA binding and nicking activities of RepC are required for pT181 DNA unwinding by PcrA. Making use of the different epitope tags present on the PcrA and RepC proteins, we found that His-PcrA was retained on an amylose resin to which MBP-RepC was bound. These results suggest a direct physical interaction between PcrA and RepC. We have also overexpressed and purified the PcrA3 mutant protein as a His-6 fusion. Studies are in progress to identify the specific biochemical activities in which the mutant PcrA3 protein may be defective.

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# COMPARATIVE CHARACTERIZATION OF *repABC* TYPE REPLICONS OF *Paracoccus pantotrophus* COMPOSITE PLASMIDS

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The *repABC* replicons have an unusual structure, since they carry genes coding for partitioning (*repA*, *repB*) and replication (*repC*) proteins, which are organized in an operon. The origin of replication (*oriV*) of these replicons has not been experimentally localized but it is suggested that it might be present within the coding sequence of the *repC* gene (1, 4). Recent studies have demonstrated that *repA*, *repB* and the *cis* required partitioning site (located downstream of the *repC* gene) constitute a functional system for the active partitioning of a *repABC*-type replicon – pTAV320 (2). Moreover, the results of studies by Ramírez-Romero *et al.* (4) showed that the protein product of the *repA* gene of another *repABC* replicon (p42d) was recognized as a negative regulator of the operon. Therefore, like the homologous A-type proteins of other plasmid partitioning systems, RepA (which is also a *trans* acting incompatibility factor) plays important regulatory functions. A search for other regulatory elements has led to the identification of two incompatibility determinants (*inc*). One of them is located within the intergenic sequence between *repB* and *repC* (probably involved in regulation of expression of *repC* gene) and the second one is situated downstream of the *repC* gene (the mentioned above partitioning site) (2, 5). So far the presence of these compact bi-functional *repABC* modules has been reported only within: (a) the megaplasmids of the Rhizobiaceae, (b) a linear chromosome of *Agrobacterium tumefaciens* C58 (7) and (c) the cryptic, composite 107 kb plasmid pTAV1 of *Paracoccus versutus* (its mini-replicon pTAV320 is the most divergent member of the *repABC* family).

We have carried out studies aimed at determining the distribution of the *repABC*-type replicons within genomes of several strains representing 11 species belonging to the genus *Paracoccus*. We found (by hybridization and PCR analysis), that *repABC* replicons occur only in the group of pTAV1-like plasmids: pKLW1, pHG16-a, pKSW2 and pPAN1 (with size approx. 70-100 kb), harbored by different strains of *P. pantotrophus*. All of the studied *P. pantotrophus* plasmids proved to be composite ones coding two different replication systems (types *repABC* and *repC'*) homologous to the replication systems of *P. versutus* plasmid pTAV1 characterized earlier (1). Two of the studied plasmids (pKLW1 and pWKS2) gave the same restriction pattern after digestion with various restriction enzymes and were thus considered identical. The *repABC*-type replicons of pKLW1, pHG16-a, and pPAN1 were cloned in the form of mini-replicons (pKLW200, pHGW200 and pPAN200, respectively) and characterized. All of them, like pTAV320, were stably maintained and had the same host range (*Paracoccus* spp., *Rhizobium* sp., *Agrobacterium* sp., *Rhodobacter* sp., but not *E. coli*).

In order to analyze the sequence diversity of the *repABC* paracoccal replicons we sequenced their *inc* regions and *repC* genes. Based on the obtained sequences we made a comparison using available data bases. This allowed the identification, within 101 kb

plasmid pRS241d of *Rhodobacter sphaeroides* 2.4.1 (*www.rhodobacter.org*), of an additional sequence highly homologous to the paracoccal *repABC* replicons. Phylogenetic analysis, based on amino acid sequences of RepC proteins, revealed that the replicons of *Paracoccus* spp. and of *R. sphaeroides* constitute a distinct evolutionary branch of *repABC* replicons.

We found that all the mentioned above incompatibility determinants of pTAV320 express *inc* phenotype towards pKLW200 and pHGW200. The nucleotide sequences of the *inc* regions of these plasmids demonstrate a high degree of identity. Interestingly, the sequence of the *repC* gene of pHGW200 and pKLW200 is conserved to a lesser degree than in the case of mini-replicon pPAN200, which is compatible with pTAV320. Turner *et al.* (5) proposed that the distinct *repC* groups of *repABC* replicons might correspond to different plasmid incompatibility classes. Based on this assumption *repABC* plasmids of *Paracoccus* spp. should be classified to the same incompatibility group. However, our results indicate that incompatibility among *repABC* replicons is a far more complex phenomenon and that all suppositions regarding this subject should be preceded by detailed sequence analysis of *inc* determinants.

Although the role of *incI* (*igs*) of *repABC* replicons is still puzzling, the *inc2* of pTAV320 was shown to carry a *cis* required partitioning site. We have previously shown the ability of the partitioning protein RepB of pTAV320 to bind to two short repeated sequences (R1 and R2) adjacent to the terminal part of the *repC* gene (*inc2*). The R1 and R2 sequences show similarities to the partitioning sites of several bacterial chromosomes, e. g. *Bacillus subtilis* (2). Such repeats were not found within rhizobial *repABC*-type sequences but are highly conserved in the paracoccal composite plasmids and in a novel *repABC* replicon present in the genome of *R. sphaeroides*. The presence of these sequences is therefore specific for the group of phylogenetically related plasmids occurring in paracocci and *R. sphaeroides* (α-3 subgroup of Proteobacteria), and may constitute an important element allowing the preliminary distinguishing of non-rhizobial *repABC*-type replicons. The results obtained in this study indicate, however, that the sequence of the gene *repC*, which is the strongest conserved area in all of the described here replicons (including the replicon present in the genome of *R. sphaeroides*) can be used as a specific probe allowing the identification of such replicons. To summarize we can state that incompatibility studies allowed to distinguish two different incompatibility groups designated IncABC1 (pTAV1, pKLW1, pHG16-a) and IncABC2 (pPAN1 and probably *repABC* replicon of plasmid pRS241d of *Rhodobacter sphaeroides* 2.4.1).

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## COPR BINDS AND BENDS ITS TARGET DNA: STRUCTURE AND SEQUENCE OF THE DNA IN THE COPR-DNA COMPLEX

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Plasmid pIP501 encoded transcriptional repressor CopR is one of the two regulators of plasmid copy number. Previous data suggested that CopR is a HTH protein belonging to a family of 578 HTH proteins (termed HTH 3-family). Only a very limited number of proteins in this family, among them λ c1 repressor, 434 c1 repressor and P22 c2 repressor have been characterized in detail so far. Previously, a CopR structural model was built based on structural homologies to the 434 c1 and P22 c2 repressor and used to identify amino acids involved in DNA binding and dimerization. Site-directed mutagenesis in combination with electrophoretic mobility shift assay (EMSA), dimerization studies and CD measurements verified the model predictions.

In this study we used hydroxyl radical footprinting and fluorescence resonance energy transfer (FRET) measurements to obtain detailed information about the structure of the DNA in the CopR-DNA complex. Our results show that the DNA is bent gently around the protein, comparable to the bending angle of 20-25° observed in the 434 c1 repressor-DNA complex and the λ c1 repressor-DNA complex. The shape of CopR dimers as determined by sedimentation velocity experiments is extended and accounts for the relatively large area of protection observed with hydroxyl radical footprinting.

A combination of *in vitro* selection (SELEX), EMSA studies with mutated operator sequences and site-directed mutagenesis was used to obtain informations about the spacer sequence between the two DNA binding sites (5'CGTG) in the CopR operator. Preliminary data suggest that the spacer has to be AT rich or must contain at least one pyrimidine/purine step which is in accordance with the bendability of the operator upon CopR binding.

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# COMPLETE NUCLEOTIDE SEQUENCE AND CHARACTERIZATION OF REPLICATION AND STABLE MAINTENANCE FUNCTIONS OF A HIGHLY DISSEMINATING PLASMID ENCODING THE CTX-M-3 β-LACTAMASE

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Several *Enterobacteriaceae* strains (*Klebsiella pneumoniae, K. oxytoca, Serratia marcescens, Escherichia coli, Citrobacter freundii, Enterobacter cloacae, Morganella morganii*) producing a novel type of an extended spectrum β-lactamase, CTX-M-3 (1), were collected by the clinical laboratory in Warsaw, Poland. The CTX-M-3 type β-lactamase production was correlated with the presence of the ~90 kb conjugative plasmid that disseminated in a very short period of time among the listed bacterial species (2) in 15 hospitals all over Poland (3).

The pCTX-M-3 plasmid was subjected to characterization by the whole nucleotide sequencing approach. It is 88254 bp in size and contains 100 ORFs identified by the Glimmer 2.0 program trained on the *E. coli* genomic sequence. 33 ORFs have homologues of known function, 49 ORFs have homologues of unknown function and 18 ORFs are pCTX-M-3 specific. Among the ORFs of unknown function there may be some involved in the plasmid spread since no ORFs encoding proteins involved in the mating pair formation could be identified by homology searches. With respect to the drug resistance, in addition to the $bla_{CTX\text{-}M\text{-}3}$ gene, the plasmid also contains the $bla_{TEM\text{-}1}$ and the integron-specified resistance to sulfonamides, aminoglycosides and ethidium bromide.

The nucleotide sequence analysis indicates that the pCTX-M-3 contains the IncL/M replicon. However, the pCTX-M-3 demonstrates much broader host-range than typical IncL/M replicons. It also transfers and stably maintains in *Pseudomonas spp, Ralstonia eutropha* and *Agrobacterium tumefaciens*. The IncL/M minireplicon of pCTX-M-3 was cloned and analyzed using the incompatibility assays to determine whether it constitutes the only replication system of pCTX-M-3. Unidirectional incompatibility of pCTX-M3 towards IncP-1 plasmids was also investigated. The pCTX-M-3 contains homologues of NR1/R100 *pemIK* (99% aa identity) and *stbAB* (45% aa identity) stability systems. The respective regions of pCTX-M-3 were cloned into unstable pOGO4 plasmid based on P7 replicon and their stabilization activities were tested.

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## INTERACTIONS BETWEEN THE pMV158-ENCODED RepB PROTEIN AND ITS DOUBLE-STRANDED ORIGIN OF REPLICATION

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The streptococcal plasmid pMV158 replicates by an asymmetric rolling circle mechanism (2). The plasmid-encoded protein RepB (24.2 kDa) is involved in the initiation of plasmid replication by interaction with the double-strand origin of replication, *dso*. Initiation of replication is achieved upon RepB-mediated cleavage of the sequence 5'-TACTACG/AC-3' (being / the phosphodiester bond cleaved by RepB), which leaves a free 3'-OH end where host-encoded proteins participate in the elongation of the nick. Cleavage is mediated by the Tyr99 residue of RepB (5, 6). We have defined two loci within the pMV158-*dso* (3), namely the *bind* region containing a set of three 11-bp directly repeated sequences (DR), and the *nic* region harbouring the specific sequence cleaved by RepB. Purified RepB protein binds specifically to the DR, which are located 84 nucleotides downstream of the nick site and in phase with it. These findings pose the question on how RepB could be transferred from the DR to the nick site, and a strong DNA bend, mediated by RepB was postulated (1).

We have now observed the existence of a new set of two 7-bp DR that are located very close (14 bp apart) to the nick site. These newly defined DR are different from those located at the *bind* region, and they have been termed UDR (for upstream direct repeats), in contraposition to the formerly defined three DR, which are now termed DDR (for downstream direct repeats). The role of the UDR has been investigated, and found to constitute a secondary RepB-binding site. RepB is able to bind to a 120-bp dsDNA fragment that contains the nick sequence and the UDR as well as to a 123-bp dsDNA fragment having only the DDR. Through band-shift experiments, we have observed that RepB exhibited an affinity that is 500-fold higher for the DDR than for the UDR, and tenfold higher for the UDR than for unspecific DNA. UDR and DDR compete for binding to the protein, indicating the existence of a single RepB domain involved in the recognition of both regions. Hydroxyl radical footprints generated by RepB bound to the DDR showed that the protein binds to the repeated sequences on the same face of the DNA helix. In addition, hydroxyl radical footprinting using the 120-bp dsDNA fragment (containing the UDR and the *nic* region) showed that the protein also binds to a region close to the nick sequence that includes (but not exclusively) the UDR. Methylation protection experiments using this 120-bp DNA fragment showed that RepB contacted with the guanosine residues of the nick sequence, as well as with some Gs of the region protected against cleavage by hydroxyl radical. Our working hypothesis is that the UDR have an important role in the proper positioning of RepB near the nick site.

Comparison of the *nic* and *bind* regions of all plasmids of the pMV158 family allowed us to observe that they all have a conserved *nic* sequence which is always located on a terminal or an internal loop within a secondary structure. Sequence differences were mainly found at the *bind* region. This is consistent with the notion that replication

specificity is provided by the *bind* region, whereas cleavage is replicon-independent (4). *In vitro*, RepB is able to cleave supercoiled or single-stranded DNA of plasmids of the same family. Accordingly, we have found the existence of putative domains in Rep proteins of the pMV158 family involved in cleavage (the more conserved N-terminal moiety), oligomerization (a Leucine-zipper motif), and DNA-binding domain (the more divergent C-terminal moiety).

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# INTERACTIONS OF THE TRANSCRIPTIONAL REPRESSOR CopG WITH ITS TARGET DNA

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CopG is a transcriptional repressor protein involved in the copy number control of the streptococcal plasmid pMV158. The genes encoding CopG and the initiator of replication, RepB, are co-transcribed from a single promoter $P_{cr}$. CopG binds to a DNA region that spans about 50 bp, and that includes the $P_{cr}$ promoter. The center of the CopG target contains a 13-bp pseudo-symmetric element (SE), both of whose half–sites are, in turn, constituted by a palindromic sequence. Purified CopG protein is a homodimer of 5.1 kDa per subunit, with a Stokes radius of 16 Å. The crystal structure of CopG has shown that the protein displays a homodimeric ribbon-helix-helix arrangement. Co-crystal structure of CopG in complex with double stranded DNA (oligonucleotides of either 19-bp or 22-bp) containing the SE, showed that two CopG dimers bind, on the same face of the DNA, to two successive helix turns, interacting with specific bases in the major groove and inducing a bend of about 60°. Electrophoretic mobility shift assays (EMSA) together with hydroxyl radical footprinting analyses indicate that several molecules of the protein bind to the same face of the DNA in a highly cooperative way, resulting in formation of, at least, four different CopG-DNA complexes. In the slowest-migrating complex, the DNA region contacted by CopG comprises four consecutive regions of the major groove facing the protein, including the two at the SE and one more at each side of this element. To investigate the contribution of both the SE and the adjacent DNA sequences to the affinity of binding by the protein, we have constructed a set of 55-bp oligonucleotides in which 1, 2 or 3 of these four CopG-facing major groove regions had been substituted by a random sequence. Another set of mutant target DNAs contained single base changes at critical bases contacted by CopG at (or closed to) the SE (as seen in the cocrystals). The complexes formed by the binding of CopG to all these mutant DNA sequences have been analyzed by EMSA. In addition, we have estimated the relative affinity of CopG for these operator mutants DNAs compared to the wild type operator. The results of these assays allowed us to conclude that the SE *per se* is not sufficient to provide specific and high-affinity binding of CopG. A model will be proposed that assumes that a stable complex is generated on the specific DNA upon binding of four CopG dimers, two of them to both half-sites of the SE, and the other two to the right and left of the SE. This nucleoprotein complex would be stabilized through CopG protein-protein interactions.

**Roles of Dimerization Surfaces of an Initiator Protein in Replication Control**

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The *pir* gene of plasmid R6K encodes two in-frame polypeptides that are designated, according to their molecular masses (in kDa), as $\pi^{35.0}$ and $\pi^{30.5}$. These variants have distinct biological properties; $\pi^{35.0}$ protein activates replication of the γ origin (*ori*) of plasmid R6K while $\pi^{30.5}$ inhibits replication. The multiple regulatory activities of π variants in replication depend on their abilities to bind a series of seven, tandem, 22-bp Directed Repeats (DRs) within the γ *ori* of R6K. It was proposed that the inhibition of replication is due to competition between dimers of $\pi^{30.5}$ and monomers (activators) of $\pi^{35.0}$ for binding to DRs. A histidine-tagging approach was adopted to purify derivatives of $\pi^{35.0}$ and $\pi^{30.5}$. The presence of his-tags allows for the use of a uniform purification procedure for these proteins that would, otherwise, exhibit distinct chromatographic properties. His-$\pi^{35.0}$ forms dimers in solution and his-$\pi^{30.5}$ is also dimeric even though it lacks a leucine zipper-like domain. However, dimers of his-$\pi^{30.5}$ are less stable in comparison to dimers of his-$\pi^{35.0}$ and are held together by a domain where numerous copy-up substitutions map. One of the copy-up variants (*pir*200, F107S) was studied, here, in detail. The F107S substitution destabilizes π dimers and its effect is more dramatic on his-$\pi^{30.5}$ in comparison to his-$\pi^{35.0}$. The mutation also reduces the DNA binding activity and the potency of his-$\pi^{30.5}$•F107S in inhibiting replication. To determine the replication function of his-$\pi^{35.0}$, alone, we obtained a M36A^M38A substitution in his-$\pi^{35.0}$. This alteration prevents the translation of $\pi^{30.5}$. His-$\pi^{35.0}$ •M36A^M38A cannot stimulate replication but it inhibits replication dependent on wt $\pi^{35.0}$. The altered protein forms more stable dimers and binds DNA solely as a dimer whereas his-$\pi^{35.0}$ binds as a monomer as well as a dimer. Introducing the F107S substitution destabilizes dimers of the his-$\pi^{35.0}$•M36A^M38A mutant, simultaneously reducing its potency to inhibit replication. However, the addition of F107S does not reverse/suppress the inability of his-$\pi^{35.0}$•M36A^M38A to activate replication even though it allows the protein to bind to DRs as monomers. The implications of these findings will be discussed.

## π PROTEIN-DEPENDENT AND π-INDEPENDENT TRANSITIONS FROM "CLOSED" TO "OPEN" COMPLEXES AT THE γ ORI OF PLASMID R6K

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The stimulation of DNA strand opening ("open" complex formation) in the R6K γ origin (*ori*) requires monomers of the plasmid-encoded π protein. *In vitro,* the open complex forms in the absence of host proteins and experiments will be presented that examine the possible effects of ATP, $Mg^{++}$ and temperature on this reaction. First, we will demonstrate that, although ATP stimulates the formation of the open complex, its hydrolysis does not appear to be required as a non-hydrolysable ATP analog, AMP-PCP, can also stimulate open complex formation. Although π lacks an identifiable ATP-binding motif, UV-induced cross-links between π and ATP can be observed *in vitro.* Additionally, we will show that, in the absence of ATP or $Mg^{++}$, π enhances the reactivity, to $KMnO_4$, of specific bases within seven, 22-bp, tandem Directed Repeats (DRs). The reactivity to $KMnO_4$ is restricted to only one DNA strand and occurs even at 0° C which indicates the phenomenon of DNA bending rather than DNA strand separation. These observations are in contrast to what we observed in the A+T-rich region where ATP (in the presence or absence of $Mg^{++}$) and higher temperature stimulate open complex formation. These and other data indicate that ATP/$Mg^{++}$ are not needed for π binding. Rather, ATP stimulates the remodeling of πDNA complexes as evidenced by the transition in DNA structure from a "closed" complex to an "open" complex. Although π is bound to DRs, DNA opening occurs at the nearby A+T-rich segment of γ *ori* and lies in the vicinity of the start sites for leading strand synthesis. Strikingly, this opening can occur in the absence of π (π-independent opening) when sufficiently high temperatures and supercoiled DNA are used in the reactions, although, at any temperature tested, the presence of πprotein enhances the number of DNA molecules engaged in the opening process.

Dimeric Isomers of Replication Initiator: DNA Binding Activities and Regulatory Roles in Replication and Transcription

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Monomers of π protein activate the replication of plasmid R6K while oligomers repress transcription of their own gene, *pir*, and inhibit replication. These activities are achieved *via* interactions of π with distinct targets that have, in common, a TGAGR core DNA sequence. Using purified, his-tagged derivatives of the protein (his-π•wt) and three hyperactive variants (his-π•F107S, his-π•P106L^F107S and his-π•S87N), we reveal elevated replication activities of the variants *in vivo* and *in vitro*. The elevated activity of the hyperactive variants, in comparison to his-π•wt, correlates with the elevated monomer-to-dimer ratio of the π species and the propensity of monomers to open the A+T-rich region present in the γ origin (*ori*) of R6K. π monomers would assemble into an oligomer on DNA comprised of seven, tandem, 22-bp Direct Repeats (DRs) in which each repeat contains a TGAGR motif (→→→→→→→). Dimers are implicated in the inhibition of replication and (auto) repression of the *pir* gene. We have proposed that an asymmetric isomer of π dimer can bind to a γ *ori* repeat while a symmetric isomer of π dimer binds to half-sites that occur in the *pir* gene operator (→•←). Herein, we set out to test this model by asking whether his-π•wt and his-π•copy-up variants differ in isomerization properties. This analysis was done by designing DNA probes containing inverted (→•←, IR) direct (→•→, RD) or everted (←•→, RE) permutations of half-sites and using these probes in electrophoretic mobility shift assays with π. A computer simulation predicted that an intrinsic bend occurs within each probe at the spacer region (•separating the IR (→•←), RD (→•→), and RE (←•→) half-site permutations. π dimers (all variants) show remarkable flexibility as they can bind and bend all three DNA probes. Hyperactive π variants, however, exhibit varying degrees of reduced binding (but not bending) to all three DNA probes in comparison to his-π•wt. Because we did not observe a correlation between the reduced DNA binding and the ratio of monomeric-to-dimeric π species, we propose that dimers of wt π and dimers of copy-up variants differ, structurally. We also examined π-dependent intra-molecular and inter-molecular interactions between DNA probes containing 22-bp DRs. Such π-mediated pairing (also known handcuffing) is believed to inhibit replication. The handcuffing abilities, in cis and in trans, of his-π•wt and copy-up π dimers are, however, very similar. The data support our working model that dimers of π protein can isomerise on the DNA into either head-to-head (π><π), head-to-tail (π□π>) or tail-to-tail (<ππ>) assemblies.

# ANTAGONISTIC BINDING EFFECT OF OLIGOMERS OF A REPLICATION INITIATOR PROTEIN (π) TO THE DIRECT REPEATS IN PLASMID R6Kγ ORI

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π protein controls replication in the γ origin (*ori*) of the plasmid R6K. At low levels, π is able to activate γ *ori*; however, at high levels, π inhibits replication. It has been hypothesized that the binding of π monomers or dimers (respectively) to the seven, tandem, 22-bp Directed Repeats (DRs) of γ *ori* result in those antagonistic activities. This hypothesis is supported by the following: (1) Both monomers and dimers can bind to a DNA probe that contains a single DR sequence. (2) π monomers can stimulate DNA strand melting at an A+T-rich region that lies in the vicinity of the DRs, while π dimers cannot. (3) Hyperactive variants of π possess an increased ratio of monomers to dimers in comparison to wt protein, and (4) π variants, for which monomer binding to DRs cannot be detected, fail to stimulate replication. Dimers of these proteins can still bind to DRs explaining the dominant-negative phenotype of these mutants. To expand on these prior observations, we decided to investigate the interactions of π monomers and π dimers with the DRs. Comparative binding studies with the aforementioned π variants (wt, hyperactive and dominant-negative) will be presented. Our results suggest that π monomers possess a higher affinity for the DRs in comparison to π dimers and (monomers) bind cooperatively, saturating all seven DRs. In contrast to πmonomers, a π dimer bound to a DR appears to sterically hinder the binding of another dimer (but not a monomer) to neighboring DRs. We will discuss a possible role for the competitive binding of monomers versus dimers in the activation and inhibition of γ *ori* replication by π protein.

# A PROMOTER FOR *OOP* RNA SYNTHESIS HAS A ROLE IN REPLICATION OF PLASMIDS DERIVED FROM BACTERIOPHAGE LAMBDA

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Transcription initiated at the bacteriophage lambda $p_o$ promoter gives a short RNA, called *oop* RNA. Early studies led to a proposal that this transcript plays a role in the initiation of lambda DNA replication. In fact, the $p_o$ promoter is located in the lambda replication region and it was suggested that *oop* RNA may be a primer for replication proceeding leftward from *ori*-lambda. However, since *in vitro* experiments demonstrated that primers for lambda DNA replication are produced by the *dnaG* gene product (DnaG primase) and subsequent *in vivo* studies indicated that *oop* RNA is an antisense RNA for the λ *c*II gene expression, the above mentioned hypothesis has fallen into oblivion. Nevertheless, we demonstrate that the $p_o$ promoter plays a role in lambda DNA replication, indeed. We found that lambda plasmids bearing a mutation that inactivates $p_o$ occur in *Escherichia coli* cells in a copy number significantly lower than wild-type lambda plasmids. Amplification of lambda $p_o^-$ plasmids during the relaxed response was less efficient relative to λ $p_o^+$ plasmids suggesting less frequent initiation of replication from *ori*-lambda in the absence of transcription from $p_o$. This suggestion was confirmed by measurement of incorporation of [$^3$H]thymidine into lambda plasmid DNA during pulse-labeling experiments. Therefore, we propose that transcription from the $p_o$ promoter stimulates replication initiation at *ori*-lambda, as suggested a long time ago, however, contrary to that suggestion, we assume that the process of $p_o$-initiated transcription *per se* but not the transcription product (*oop* RNA) might play a role at early steps of λ DNA replication.

# *PARTITION AND STABILITY*

## ORAL PRESENTATIONS

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**77** The highly conserved TldD and TldE proteins of *Escherichia coli* are involved in Microcin B17 processing and in CcdA degradation. Noureddine Allali, Hassan Afif, Martine Couturier and Laurence Van Melderen

TIME LAPSE PHOTOMICROSCOPY OF P1 PLASMID PARTITION IN LIVING CELLS.

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The fate of the P1 plasmid of *Escherichia coli* was followed by time-lapse photomicroscopy. A GFP-ParB fusion marked the plasmid during partition (segregation) to daughter cells at slow growth rate. GFP-ParB binds specifically to the P1 partition site *parS*. Like the wild-type ParB protein, the fusion has the unique property of loading onto the DNA at this specific site and spreading out onto adjacent sequences to form a region coated with multiple bound copies of the protein. This phenomenon is responsible for gene silencing around the *parS* site, but here it serves to produce a bright focus consisting of many fluorescent molecules that marks the position of the plasmid in the growing cells. This has allowed us to follow the fate of the plasmid as cells grow and divide on the microscope slide. The process is fundamentally different from that previously inferred from statistical analysis of fixed cells. A focus of plasmid copies is captured at the cell center. Immediately before cell division, the copies eject bidirectionally along the long axis of the cell. Cell division traps one or more plasmid copies in each daughter cell. These copies are free to move, associate and disassociate. Later, they are captured to the new cell center to re-start the cycle. Null P1 *par* mutants are neither captured nor ejected. A single focus is randomly placed and ends up in one daughter cell. A dominant negative ParB protein forms a plasmid focus that attaches to the cell center but never ejects. It remains captive at the center and blocks host cell division. The cells elongate. Eventually the intact focus is pushed to one side and the cells divide simultaneously in several places. This implies that the wild-type plasmid imposes a regulatory node on the host cell cycle, preventing cell division until its own segregation is completed.

**THE INTERACTION OF ParA AND ParB PROTEINS DURING P1 PLASMID PARTITION IN *Escherichia coli.***

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Faithful partition of P1 plasmids in *Escherichia coli* requires the action of two plasmid-encoded proteins, ParA and ParB, which act via a centromere-like site called *parS*. Many circular as well as linear plasmid and bacterial chromosomes encode homologues of ParA and ParB. We have been studying the steps in the partition process by examining the interactions of these proteins with DNA and with each other. ParB is the key centromere-binding protein – we have shown that an initial complex forms when a dimer of ParB, aided by a DNA bend induced in the center of *parS* by *E. coli* IHF, binds. Multiple dimers of ParB load onto this complex, and *in vivo* evidence indicates that many molecules of ParB then associate with the plasmid to form a large nucleoprotein complex. ParA, an ATPase, acts on this complex to localize the plasmid inside cells. However how ParA accomplishes this localization is unknown. Our recent results indicate that ATP hydrolysis is required for the localization activity or activities of ParA. Mutant ParA proteins that can bind but not hydrolyze ATP are ineffective at partition although they retain the ability to act as repressors of the par operon. Our data also suggest that the large complexes of ParB that form at *parS* can sequester multiple copies of the plasmid into one complex. We have been testing the idea that one role of ParA is to separate pairs or clumps of plasmids following replication. Certain mutations in the ATP binding site of ParA appear to be defective in this reaction. We have analyzed these mutations both *in vivo* and *in vitro*. We have also used circular dichroism and limited exposure to proteases to probe the structure of mutant and wild-type par proteins. Our experiments suggest (i) that the N-terminus of ParB contains regions that interact with itself during the oligomerization reaction and also with ParA, (ii) that ParB alters its conformation after the initial dimer binds to *parS*, presumably prior to or coincident with oligomerization, (iii) that ParA interaction with the N-terminus of ParB disrupts the oligomerization reaction, and (iv) that ATP hydrolysis is required for this interaction.

# THE ParFG PARTITION PROTEINS

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The active partition module of the multidrug resistance plasmid TP228 consists of two genes, *parF* and *parG*, and essential upstream sequences which are likely to include the partition site. The ParG protein (8.6 kD) is dimeric in solution as revealed by chemical cross-linking, analytical ultracentrifugation and mass spectrometric analyses. ParG oligomerization can also be conveniently monitored in a bacterial two-hybrid assay *in vivo*. ParG is a DNA binding protein: four 17-19 bp direct repeats and a related inverted repeat located upstream of *parFG* are putative ParG binding sites. The ParF protein (22 kD) is a member of the ParA superfamily of ATPases which are involved in bacterial plasmid and chromosome segregation, and cell division. Chemical cross-linking and analytical ultracentrifugation experiments suggest that ParF can oligomerize in solution. Furthermore, purified ParF and ParG proteins interact *in vitro* as visualized by affinity pull-down assays; this interaction is enhanced by ATP. ParFG complex formation is also detectable on native gels and by cross-linking experiments, and also in a bacterial two-hybrid system *in vivo*. Although these interactions do not require the presence of partition site DNA, ParF also promotes the interaction of ParG with the putative partition site. The three-dimensional organization of ParG is under investigation using nuclear magnetic resonance (NMR) techniques.

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## THE REGULATORY PROTEIN ω FROM THE BROAD-HOST RANGE *STREPTOCOCCUS PYOGENES* PLASMID PSM19035: ANALYSIS OF BINDING TO OPERATOR DNA WITH ONE TO FOUR HEPTAD REPEATS

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pSM19035-encoded ω protein regulates transcriptional levels of genes required for control of plasmid copy number and stable inheritance. The ω protein, that has $T_m$ values between ~ 43 and ~ 78°C depending on the ionic strength of the buffer, is mainly α-helical (42%) (1). The ω protein belongs to the structural superfamily of MetJ/Arc repressors featuring a ribbon-helix-helix DNA binding motif with the β-ribbon located in the major groove of operator DNA (2). The ω protein cognate site were mapped upstream the promoter regions of the *cop*S (*P*cop1 and *P*cop2), δ (*P*δ) and ω (*P*ω) genes (3). These promoters are embedded in a set of either seven copies of a 7-bp direct repeat and two inverted repeats [*P*δ, $(\longrightarrow)_7(\longleftarrow)_2$] or a block consisting of two 7-bp direct repeats and one 7-bp inverted repeat plus one additional invert repeat downstream of the block [*P*ω, $[(\longrightarrow)_2(\longleftarrow)]_2(\longleftarrow)$] and one additional invert repeat [*P*copS $[(\longrightarrow)_2(\longleftarrow)]_3(\longrightarrow)$]. Gel retardation assays, DNase I and hydroxyl radical footprinting, surface plasmon resonance, and spectroscopic evidence demonstrate binding of ω protein to DNA segments containing two, three and four 7-bp repeats with sequence 5'-$^{A}/_{T}$ATCAC$^{A}/_{T}$-3' in different orientation. The ω protein binds poorly to one a heptad, but binds with high specificity to two unspaced heptad. Protein-DNA complex formation is, however, increased with DNA containing three or more heptad repeats. Spacing between the two heptads, by 1-, 3- or 7-bp, drastically impairs the affinity of ω protein. Severely reduced ω binding activity to two directly oriented heptads was demonstrated for the substitution of the TCAC motif of one heptad by any other nucleotide. Our results show that by protein-protein interaction ω oligomers gain a certain degree of flexibility that enable complex formation with direct and inverted 7-bp binding sites.

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**Prokaryotic Analogue of the Eukaryotic Spindle Apparatus**

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The partitioning (*par*) locus encoded by plasmid R1 actively segregates DNA by an unknown mechanism. Here we show that the ParM ATPase encoded by *par* forms dynamic actin-like filaments. Filament formation depended on the other components encoded by *par*, ParR and *parC*. ATPase defective ParM mutant proteins exhibited abnormal filament morphology and turnover and did not support plasmid DNA segregation. In vitro, ParM polymerization was ATP dependent and its ATPase activity exhibited strong cooperativity. Filaments formed in the presence of ATP were transient and depolymerization required nucleotide hydrolysis. These observations strongly support that polymerization of ParM functions to segregate plasmid DNA prior to cell division. Thus, we provide evidence of a prokaryotic analogue of the eukaryotic mitotic spindle apparatus.

## THE STRUCTURE OF THE KID TOXIN: LOCALIZATION OF FUNCTIONAL REGIONS AND COMPARISON WITH THE CCDB TOXIN AND WITH OTHER HOMOLOGOUS TOXINS.

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The Kid toxin and its anti-toxin, the Kis protein, are the two components of the conditional killer stability system *parD* of plasmid R1 (Bravo *et al.*, 1997; 1998). We have determined the crystal structure of Kid toxin at 1.4 Å resolution. This structure reveals a two-fold symmetric dimer. A monomer of Kid has overall dimensions of approximately 30Å x 30Å x 30Å and the main feature of its fold is a 7 strand, twisted antiparallel β-sheet of which the first and last three strands almost fold up to form a β-barrel. Alignment with Kid homologous toxins found in the chromosome of many eubacteria, and analysis of non-toxic mutants of Kid suggest that the interaction surface of Kid with its cellular target incorporates the loop between β1 and β2 (residues 10-22), and possibly extends around the molecule to residues in strand β5. The structure of Kid closely resembles the DNA gyrase-inhibitory toxin protein CcdB (Loris *et al.*, 1999) from the *E.coli* F plasmid despite the lack of any notable sequence similarity. However mutation studies have suggested a target interaction surface on CcdB that involves the final three residues of its C-terminal helices (Bahassi, *et al.*,1995) i.e. the opposite face of the protein to that proposed for Kid-target interaction. Furthermore, the size and orientation of the loops in Kid that are implicated in its toxicity are not maintained in CcdB.

Interactions between the Kis and Kid proteins are required to form the repressor of the *parD* system (Ruiz-Echvarría *et al.*, 1991a; 1995). Therefore mutants in Kid leading to de-regulation give information on these interactions. Analysis of these mutants suggests that the highly flexible loops extending on either side of the protein formed by residues Ser47 to Arg53, remote from the two-fold axis, may have functional significance in anti-toxin binding. This region is equivalent to the flexible loop of CcdB (Ser38-Arg 48) that has been implicated in binding the CcdA antitoxin (Van Mellderen et al., 1996). As some of the mutants interfering with the co-regulatory activity of Kid are adjacent to non-toxic mutants, it is possible that the Kid-target and Kis binding sites overlap and that binding of Kis thus antagonizes the toxic effect of Kid. In Kid and CcdB the location of the regions involved in interactions with the anti-toxins are conserved while the elements involved in interactions with the cellular target are located in different regions of these proteins.

The data presented here and elsewhere (Ruiz-Echevarría *et al.*, 1991b) strongly suggest a common origin for *parD* and *ccd* systems. As such, it is interesting to note that the *ccd* system is confined to F-like plasmids, while *parD*-like systems are widespread in eubacterial genomes (Gerdes, 2000; Mittenhuber, 1999). This suggests an evolutionary

advantage for *parD*-like systems compared to *ccd*-like systems for reasons that remain to be determined.

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# SOLUTION STRUCTURE AND DYNAMICS OF THE ANTITOXIN PARD FROM THE PLASMID RK2/RP4 KILLING MODULE

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The low copy number plasmid RK2/RP4 is stably inherited for many generations within a broad range of gram negative bacterial hosts. A major contributing factor for stabilization properties is the protein killing system encoded in the *parDE* operon on the plasmid. The gene products, ParD and ParE, comprise two small proteins whose intricate interplay ensures that only daughter cells harboring the plasmid survive within a bacterial population. The antidote protein, ParD, is short-lived and complexes the long-lived toxic protein ParE thus antagonizing its lethal effect. ParD also autoregulates its expression negatively by binding to an operator sequence in the *parDE* promoter region. In cells which have been cured from the plasmid the ParD protein is degraded faster than ParE. As a result the unopposed ParE protein causes growth retardation and cell killing. We set out to elucidate the structural components of ParD which lay the foundation for this highly concerted mechanism. Characterization of ParD has been performed using different biophysical techniques and the first solution structure of such a bacterial antidote which has been solved by multidimensional NMR-spectroscopy is presented [1, 2].

In the overall structure of the homodimeric ParD two distinct moieties can be identified (Figure 1): The protein comprises of a well structured N-terminal region and a highly flexible C-terminal domain which appears to be unstructured or in fast exchange. The structure consists of two momoneric subunits, where each polypeptide chain comprises a single β strand followed by two intertwined helices. The strands from each subunit pack side by side forming a slightly twisted antiparallel sheet. A third helix is connected to the well ordered N-terminal domain via a flexible loop region. The protein can be classified as a member of the ribbon-helix-helix fold family of DNA-binding proteins, similar to the other prokaryotic repressors Arc and MetJ and also to the recently solved crystal structure of the plasmid encoded repressor protein omega [3, 4]. From the solution structure of ParD the mode of DNA-binding can be inferred such that the short β ribbon is inserted into the major groove of the cognate DNA. Specific base contacts can be mediated by solvent exposed hydrophilic residues of the strands. Dynamic properties of the protein were investigated by relaxation measurements and revealed the same division into two structurally distinct regions of the protein.

The solution structure of ParD and the biological implications of the characteristics of the protein with respect to dimerization, ParE- and DNA-binding will be discussed.

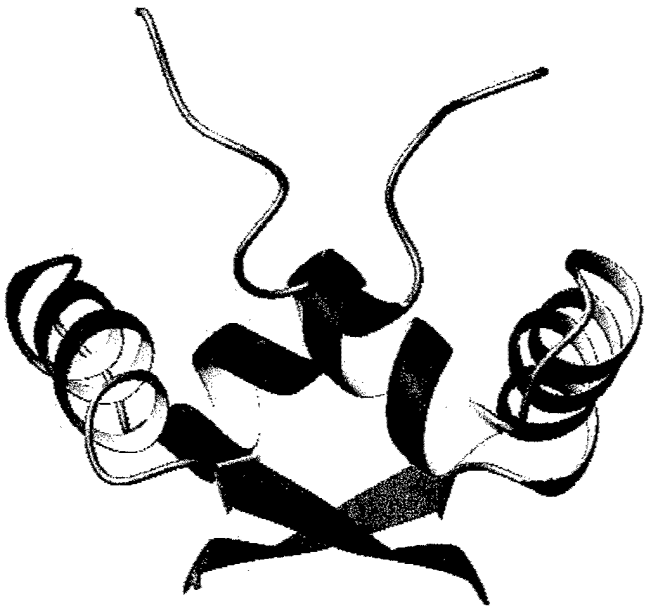

Figure 1: Ribbon drawing of the well-defined N-terminal region of the antidote protein ParD.

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# COLE1 DIMER RESOLUTION: HOW DOES SYNAPTIC COMPLEX STRUCTURE PREVENT CATASTROPHIC DIMER FORMATION?

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The accumulation of dimers poses a threat to the stable maintenance of multicopy plasmids. Dimers formed by homologous recombination accumulate rapidly due to the faster replication of dimers; a process known as a dimer catastrophe. The consequent decrease in the number of plasmid molecules in the cell increases the frequency at which plasmid-free cells arise. To counter the dimer catastrophe plasmids use site-specific recombination to restore themselves to the monomeric state. The paradigm dimer resolution system is that of ColE1, where Xer-mediated recombination occurs between *cer* sites (Summers, 1998). Consistent with its biological role, Xer-*cer* recombination is constrained to intra-molecular events, so it resolves multimers and avoids creating new ones.

Recombination at *cer* requires four host-encoded proteins: ArgR (the arginine repressor), PepA (aminopeptidase A), XerC and XerD (members of the lambda integrase family of site-specific recombinases). Although ArgR, and PepA have no direct involvement in strand exchange, they have important accessory roles in dimer resolution. At fully-constrained sites (e.g. ColE1 *cer*) they are needed to activate recombination, and XerCD is impotent unless both are present. However, for recombination at the Supersite family of *cer*-like sites, recombinase activation is not required and the constraining role for ArgR and PepA becomes apparent. In the presence of the accessory factors, Supersites are limited to intra-molecular recombination (i.e. to dimer resolution) but in the absence of either, recombination becomes unconstrained (i.e. both intra- and inter-molecular).

Two models have been proposed for the mechanism by which Xer-*cer* recombination is constrained to dimer resolution. In the Oxford model (Colloms et al., 1997) constraint is imposed by DNA topology. Formation of the synaptic complex is proposed to require inter-twining of the recombining sites; something which is energetically feasible only when the sites are on the same molecule. In contrast in the Cambridge model (Hodgman et al., 1998) the accessory proteins are the prime movers. The recombining sites are held together in the synaptic complex by protein-protein interactions which require reinforcement by the "spring clip" effect of supercoiled DNA passing between the sites. When the sites are on different molecules and the spring clip is absent, the synaptic complex is extremely unstable.

What do these models have to say about the possibility of escaping from constraint? It is difficult to see a simple way to circumvent the strong hand of DNA topology favored in Oxford, but in a strong prediction from Cambridge is that if the protein-protein interactions which hold together the sites in the synaptic complex were strengthened, constraint would be overcome. This should be possible by mutating the accessory proteins.

Using a genetic screen in which inter-molecular Xer-*cer* recombination is required to maintain antibiotic resistance we have isolated a number of dominant alleles of *argR* and *pepA* which either abolish or severely reduce the normal constraints on the system. These alleles have a detrimental effect on host fitness and are difficult to maintain in the absence of selection.

The mutations have been mapped to the 3D structures of ArgR and PepA. Two mutations in PepA map to the outward-facing surface of the hexamer. Several of the ArgR mutations cluster at or near the trimer-trimer interface of the hexamer. The possible of these changes on the stability of the synaptic complex will be discussed in the light of the Oxford and Cambridge models.

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# ARCHITECTURE OF THE ParF•G PROTEIN COMPLEX INVOLVED IN ACTIVE DNA SEGREGATION

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The mechanism by which low-copy number plasmids are delivered to daughter cells at cell division requires the concerted action of two plasmid-encoded proteins that assemble on a centromere-like site. The ternary nucleoprotein complex is thought to interact with specific host structures that direct the plasmid to targeted subcellular locations. The partition locus of the multidrug resistance plasmid TP228 in *Escherichia coli* consists of two cotranscribed genes, *parF* and *parG*, and an upstream centromere-like assembling site. This partition cassette belongs to a recently discovered subgroup of plasmid partitioning systems that are phylogenetically distinct from the P1, F and R1 prototypes. *parF* encodes a 22 kDa protein that is a member of the ParA superfamily of ATPases since it contains a conserved Walker-type ATP-binding motif. The homology shared by ParF with ParA of P1 is largely confined to the ATP-binding domain, whereas the homology of ParF to other members of the above mentioned subgroup extends throughout the entire length of the protein. The product of *parG* is a 8.6 kDa polypeptide that exhibits no homology to the ParB partition protein of P1 plasmid. A bacterial two-hybrid system based on the reconstitution of a transduction pathway has been used to show that ParF interacts with ParG *in vivo*. Affinity chromatography experiments involving GST-pull downs with purified recombinant proteins demonstrate that ParF directly binds to ParG in an ATP-dependent manner. ADP does not support the association of the two proteins. This interaction does not require the presence of the putative partition site. The ParF•ParG interaction has been further detected by the use of non-denaturing PAGE. ParG oligomerizes *in vivo* as observed by the bacterial two-hybrid analysis; gel filtration chromatography, equilibrium sedimentation and chemical cross-linking experiments unequivocally show that ParG is a dimer in solution. ParF also forms oligomers *in vivo*, and dimers and to a lesser extent higher multimers are detected in cross-linking experiments *in vitro*. When both ParF and ParG are incubated in the presence of cross-linker, at least two distinct complexes are detected. Complex formation appears to be enhanced by the presence of ATP. The putative partition site is located upstream of the *parFG* operon. Electrophoretic mobility shift assays have revealed that ParG binds specifically to this region containing repeated sequences, whereas ParF does not appear to contact the DNA directly. At limiting ParG concentrations, the putative partition site is shifted into a ladder of multiple complexes with different mobilities. However if equimolar quantities of ParF are added to the reaction under the above conditions, a discrete complex is formed. Therefore ParF affects the affinity of ParG for its cognate sequences in the putative partition region and enhances the formation of a ternary partition complex. Future studies will focus on defining the strategic interactions of the ParF•ParG complex with potential host structures.

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Common features of plasmid and chromosomal members of PARB family

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Among the many conserved features of bacterial genomes revealed by genome sequencing is a pair of proteins encoded near the replication origin *oriC* (1), which appear to be critical for proper placement of the bacterial nucleoid as bacteria grow and divide (2). These proteins termed ParA and ParB belong to the continuously growing families of plasmid and bacterial chromosome partitioning proteins (3, 4). We previously mapped functional domains in KorB of RK2 plasmid (IncP-1) (5) and similar domain analysis was performed on ParB homologue of *Pseudomonas aeruginosa*. Using the yeast two-hybrid system we mapped the domain of interaction with $ParA_{P.a.}$ to C-terminal 47aa of $ParB_{P.a.}$. The C-terminal domain also happens to be the ParB dimerization domain. The role of the C-terminus in $ParB_{P.a}$ multimerization was confirmed *in vitro* by glutaraldehyde cross-linking experiments. The localization of the dimerization domain seems to be the universal feature of ParB homologues. The importance of the H-T-H motif in DNA binding ability of $ParB_{P.a}$ was confirmed. The role of conserved the ParB BoxI will be discussed. Overproduction of $ParB_{P.a}$ in *E.coli* carrying $R751Tc^R$ (IncP-1) leads to the loss of otherwise stable plasmid suggesting interplay between plasmid and chromosomal partitioning apparatus. The C-terminal dimerization domains of $KorB_{RK2}$ and $ParB_{P.a}$ can be interchanged and are functional in the hybrid proteins *in vitro*. Both $KorB_{RK2}$ and $ParB_{P.a}$ are able to cause the instability of the plasmid with the $KorB_{RK2}$ and $ParB_{P.a}$ binding site, respectively. This reaction is dependent on the presence of DNA binding domain and dimerization domains even if the latter are heterologous.

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# FUNCTIONAL PROPERTIES OF RepA AND RepB, PROTEINS OF THE PARTITION SYSTEM OF A *repABC* PLASMID

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The replication/ partition region of the symbiotic plasmid (pSym) of *Rhizobium etli* consists of three genes *repA, B and C*, organized in an operon, plus an origin of replication and a partition site. Two *cis*–incompatibility sites have been identified, one located in the intergenic sequence between *repB* and *repC (incα)* and the second, immediately downstream of the *repC* gene *(incβ)*. The *repA* and *repB* genes encode proteins required for plasmid stability, and plasmid copy-number control. RepA also acts as a *trans*-acting incompatibility factor.

Two sigma-70 like promoters, oriented in the same direction, have been found in the *repABC* operon, one of them positioned upstream of *repA* and the other within the *repC* gene. The second promoter transcribes a RNA encoding a putative peptide of 11.22 kd. This hypothetical transcript embraces the *incβ*. The second promoter is stronger than the first. The function of this imbricate gene remains unknown but its possible role in partition or in replication is currently being evaluated.

It has been show that RepA is able to repress the transcription of the *repABC* operon. An analysis of the upstream region of *repA* showed the presence of a 14 bp inverted repeat, located between the promoter and the initial codon of *repA*. The disruption of this DNA motif suppresses the ability of RepA to repress transcription. To demonstrate that the region embracing the inverted repeat is the binding site of RepA, gel mobility shift assays were performed with purified RepA-H6 or MalE-RepA fusion derivatives and using as target DNA the upstream sequence of *repA* or the same region but lacking the inverted repeat. These experiments showed that RepA is able to bind to the DNA with the inverted repeats but not to the sequence lacking them, indicating that the RepA binding site resides within the inverted repeats. Gel mobility shift assays also showed that RepA is unable to bind *incα* or *incβ*. The RepA DNA binding activity has an absolute requirement for ATP or ADP, suggesting that ATP hydrolysis is not necessary for DNA binding.

Recently, Bartosik and coworkers demonstrated the presence of two *cis* – incompatibility sites in pTAV320, a member of the *repABC* plasmid family isolated from *Paracoccus versutus*. As in the case of the pSym of *R.etli*, one site located in the intergenic sequence between *repB* and *repC (inc*1*)* and the other, immediately downstream of the *repC* gene *(inc*2). RepB was able to bind *inc2*. Moreover, in *E. coli*, plasmids carrying this region can be destabilized in an excess of RepB. In *P. versutus*, an unstable plasmid improves its stability if it carries *inc2* and if RepA and -B are provided in *trans*. These results demonstrated that *inc2* carries a sequence with a centromere-like activity. The *inc2* of pTAV320 and *incβ* of the pSym of *R. etli* do not share sequence

similarity, but they are, however, functionally homologous: RepB of the pSym is able to bind *incβ* in gel mobility shift assays but not in the promoter region or *incα*. Also, plasmids carrying *incβ* improve their stability when RepA and RepB are provided in *trans*. Nevertheless, these plasmids do not recover the stability to the extent of the wild type indicating that something is missing in the system.

Progress has been made in determining the ability of RepA or RepB to form dimers, and tin their ability to interact with one another, using a bacterial two-hybrid system.

Oscillation of ParA of plasmid pB171 is correlated with plasmid stabilization

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Prokaryotic plasmids and chromosomes encode partitioning (*par*) loci that segregate DNA to daughter cells prior to cell division. Recent database analyses showed that all known *par* loci encode an ATPase and a DNA-binding protein, and one or more *cis*-acting regions where the proteins act. All *par*-encoded ATPases belong to two protein super-families, Walker-type and actin-like ATPases. This property was recently used to divide *par* loci into Type I and Type II loci [Gerdes, K., Møller-Jensen, J. & Jensen, R.B. (2000) *Mol. Microbiol.* 37, 455-466]. We show here that the *Escherichia coli* virulence factor pB171 encodes a double *par* locus that consists of one Type I and one Type II locus. Separately, both loci stabilized a test-plasmid efficiently. Together, the two loci mediated even more efficient plasmid stabilization. The *par* loci have a unique genetic organization in that they share a common, central region at which the two different DNA-binding proteins probably act. Interestingly, a fusion-protein consisting of the Walker-type ParA ATPase and Gfp was functional and oscillated in nucleoid regions on a timescale of minutes. ParA-Gfp oscillation depended on both ParB and *parC* but was independent of *minCDE*. Point mutations in the Walker A box motif simultaneously abolished plasmid-stabilization and ParA-Gfp oscillation. These observations suggest that ParA oscillation is prerequisite for active plasmid segregation.

Using a novel Gfp based technique, we report that *par2* of pB171 localizes plasmids predominantly at mid-cell. Based on these results we suggest that plasmid positioning is coupled to oscillation of ParA.

REGULATION MECHANISMS OF THE *PAR* POST-SEGREGATIONAL KILLING SYSTEM

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The *par* locus of the *Enterococcus faecalis* plasmid pAD1 is 404 bp and is required for the maintenance and stable inheritance of the pAD1 replicon. It has been shown that *par* stabilizes plasmid replicons by a post-segregational killing (PSK) mechanism. *Par* encodes two small RNAs of ~210 nt (designated RNA I) and ~65 nt (designated RNA II). RNA I is relatively stable *in vivo* and encodes the *par* toxin, while RNA II is the unstable antidote. Although the *par* region is not organized in a typical antisense/target arrangement, RNA II encodes regions complementary to both the 5' and 3' ends of RNA I and suppresses toxin function via an antisense mechanism. RNA I encodes an open reading frame of 33 codons, whose product is the *par* toxin. Binding of RNA II to its complementary sequences at the 5' end of RNA I interferes with translation of *fst* (faecalis plasmid stabilizing toxin), consistent with its role as an antisense regulator of the system. It is a common feature of antisense regulated PSK systems to have an inactive pool of toxin message that is processed into an active form, thereby limiting it to plasmid-free cells. A 5' truncated mutant of RNA I has been shown to be translated with a much higher efficiency indicating possible 5' processing of RNA I to an active form.

In this work, we sought to study the regulatory mechanisms of the *par* PSK system. Previous semi-quantitative northern blot analyses showed a five molar excess of RNA I, indicating that a possible regulatory mechanism in addition to RNA II controls *par*. A more sensitive quantitative RNA tool, ribonuclease protection assay, is being utilized to study this further. An accurate quantitation of endogenous levels of RNA I and RNA II is the essential first step of this study. Secondly, the regulation of this system is also being studied *in vivo* by placing RNA II downstream of a regulatable promoter in a gram-positive expression vector. Using the RNA II nisin construct *in trans* with an endogenous RNA I gene will allow us to study the effects that RNA II has on RNA I transcription and processing when the antidote is actively transcribed and when transcription is suppressed.

# A NOVEL, BROAD SPECTRUM TOXIN-ANTITOXIN MODULE FROM A MULTIDRUG-RESISTANT, CLINICAL ISOLATE OF *ENTEROCOCCUS FAECIUM*

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*Enterococcus faecalis* and *E. faecium* are now acknowledged as leading causes of hospital-acquired bacteremia, urinary tract infections and surgical wound infections. High-level gentamycin-resistant and vancomycin-resistant strains of enterococci have been reported worldwide during nosocomial outbreaks. In laboratory tests, these plasmid-encoded resistance genes can be transferred to other more pervasive bacteria such as *Staphylococcus aureus*, revealing a worrying potential route of antibiotic-resistance dissemination in the microbial community. The emergence of multidrug-resistant enterococci is also of considerable concern because it reduces the range of therapeutic options available for treatment of conditions caused by these bacteria. pRUM is a multidrug resistance plasmid found in a vancomycin-resistant clinical isolate of *Enterococcus faecium* in the USA. Bioinformatics analysis of the nucleotide sequence of pRUM (24,873 bp) led to the identification of two overlapping genes whose sizes and organization are characteristic of toxin-antitoxin systems. The expression of both genes was essential to stabilize heterologous, unstable vector plasmids; this activity was evident in both Gram-positive (*E. faecium* and *Bacillus thuringiensis*) and Gram-negative (*E. coli*) hosts. The toxic and antitoxic natures, respectively, of the Txe (toxin from *Enterococcus*) and Axe (antitoxin from *Enterococcus*) proteins were demonstrated by cloning the corresponding genes into the arabinose-inducible expression vectors pBAD24 and pBAD28, respectively. Under repressing conditions (*i.e.*, in the presence of glucose), growth of *E. coli* containing the recombinant plasmids pBAD24::*txe* or pBAD28::*axe* was normal. Under inducing conditions (*i.e.*, in the presence of arabinose), expression only of the *txe* gene resulted in extensive growth inhibition. This toxic effect of Txe (89 amino acids) was alleviated by co-expression of the antitoxin protein Axe (85 amino acids). The predicted *axe* and *txe* protein products share homology with putative proteins encoded by a diversity of similarly-organized chromosomal modules in the genomes of both Gram-positive and Gram-negative bacteria, including with proteins of plasmid pFNL10 which were recently hypothesized to act as a toxin-antitoxin system in *Francisella* sp. PhD-Doc of bacteriophage P1 is one of the most well-characterized toxin-antitoxin systems: the Axe and PhD antitoxin proteins are evolutionarily related, but the Doc protein and its homologues specified by many bacterial genomes are much more distantly related to Txe and its closest neighbors. Although the toxin-antitoxin activity of most of these modules has yet to be established, these observations suggest that the mechanism of Axe-Txe activity is widespread and functionally conserved amongst both plasmids and chromosomes in a variety of evolutionary diverse bacteria.

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REGULATION OF TOXIN EXPRESSION IN THE *PAR* POST-SEGREGATIONAL KILLING SYSTEM: TRANSLATIONAL SUPPRESSION BY STRUCTURES AT THE 5'END OF RNAI.

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The *par* stability determinant of the *Enterococcus faecalis* plasmid pAD1 is the first antisense RNA regulated post-segregational killing system (PSK) identified in a Gram-positive organism. *Par* encodes two small, convergently transcribed RNAs, designated RNAI and RNAII, which are the toxin and antitoxin of the *par* PSK system, respectively. RNAI encodes an open reading frame for a 33 amino acid toxin called Fst. Expression of *fst* is regulated post-transcriptionally by RNAII. RNAII is less stable than RNAI; therefore pAD1 must be maintained to prevent Fst-toxin expression. If a daughter cell does not receive a copy of the plasmid, RNAII levels decline allowing Fst to be expressed killing the cell. Thus, the *par* system maintains pAD1 in the host population by killing plasmid-free segregants. RNAI:RNAII interaction appears to be unique among antisense RNA systems in that the RNAs interact at multiple dispersed sites within the 5' and 3' ends of both RNAs. The initial interaction between RNAI and RNAII occurs at the 3' end of the RNAs. This unstable initial interaction is then stabilized through the stepwise interaction of two direct repeats at the 5' ends of the RNAs.

It is still unclear how RNAI remains to be translated in plasmid-free cells if it is bound to RNAII. By analogy with the other antisense regulated PSKs, a pool of "inactive" RNAI should exist which cannot be translated until processed. Structural analysis of RNAI indicates that the ribosome binding site for Fst is located within a stem-loop at the 5' end of the RNA. Additionally, another helix precedes this stem-loop and coaxial stacking of this helix and the 5' stem-loop may contribute to translational suppression of Fst. Mutational analyses indicate that the 5' stem-loop alone is sufficient to suppress Fst translation *in vitro*. Deletion of the upstream portion of this stem-loop results in a significant increase in translation levels *in vitro*. Base changes that disrupt the complementary pairing of the stem-loop have similar effects. Complementary mutations that restore the stem-loop structure reduce translation levels to that of the WT RNAI. Experiments are underway to determine what effects these mutations will have *in vivo*.

# NMR STUDIES OF ParG, A PROTEIN REQUIRED FOR ACTIVE PARTITION OF BACTERIAL PLASMIDS

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The segregational stability of bacterial low copy number plasmids is promoted by active partition which ensures that each of the daughter cells faithfully acquires a copy of the plasmid after cell division. The ParG protein (8.6 kD) is an essential plasmid-encoded component of the partition system of the multidrug resistance plasmid TP228. The ParG protein binds DNA upstream of the *parFG* genes, exists as a dimer in solution, and interacts *in vivo* and *in vitro* with ParF, the second plasmid-encoded protein in this system. The structural properties of ParG were investigated using nuclear magnetic resonance (NMR) techniques. The full backbone and sidechain NMR signal assignments were performed using $^{15}N$ and $^{15}N,^{13}C$-labelled protein samples and a conventional set of three-dimensional double-resonance NMR experiments. The presence of negative or small positive heteronuclear NOEs indicates that the N-terminal part of ParG (~30 residues) is very flexible. The analysis of NOE contacts, chemical shift indices and deuterium exchange rates suggests that this flexible tail is followed by β-structure, and that the C-terminal part of ParG forms a tightly packed α-helical structure. Part of the flexible N-terminus (residues 1-19) is cleaved *in vitro* during prolonged incubation; the possibility and functional importance of this cleavage as a regulatory mechanism *in vivo* is still to be elucidated although the N-terminal tail appears not to be required for ParG dimerization. The NMR derived data (NOE distance constraints and dihedral angle constraints) will be used for the calculation of the detailed three-dimensional structure of ParG. Combined with mutational, biochemical and biophysical analyses, structural studies of partition proteins will provide important new insights into the molecular mechanisms by which genome segregation is achieved in bacteria.

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## A ROLE FOR *KFRA*, *UPF54.8* AND *UPF54.4* IN STABLE MAINTENANCE OF INCP-1 PLASMIDS

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Conjugative IncP-1 plasmids are stably maintained in the broad spectrum of Gram negative bacteria (1). Plasmids of IncP-1α subgroup rely on different stabilization mechanisms (2) but the stability of R751 (IncP-1β subgroup) seems to depend mainly on the activities of IncC and KorB proteins, global regulators encoded in the central control operon and involved in the active partitioning process (3). Adjacent to the central control operon *korABFG incC* lies the *kfrA upf54.8 upf54.4* operon whose function is still not well understood. Although we demonstrated weak promoter activities upstream of both *upf54.8* and *upf54.4,* their expression proceeds mainly from the very strong *kfrAp*, regulated by KfrA and at least two global regulators: KorA and KorB. The *kfrA upf54.8 upf54.4* region is additionally very interesting because of the presence of two $O_B$s (classII in *kfrAp* and class III in the intergenic *upf54.8-upf54.4* region) whose roles as the putative centromere-like sites have been suggested (4,5). KfrA proteins of IncP-1 are DNA-binding proteins of unusual α-helical structure (3,5). Their extended coiled-coil domains show homology to SMC proteins and eukaryotic myosin/kinesin family. Upf54.4 has also a high α-helical content. Overproduction of $KfrA_{R751}$ strongly affects nucleoid condensation and segregation in the host cells whereas overproduction of Upf54.4 $_{R751}$ blocks cell growth and division. Purification of $KfrA_{R751}$ (342aa) confirmed its α-helical structure and ability to polymerize as was observed previously for $KfrA_{RK2}$(5). N-terminal 136aa are sufficient for dimerization *in vitro* and *in vivo*. However, the autoregulatory function of such a truncated polypeptide is highly impaired. The mutant *kfrAΔC* allele (coding for N-terminal 136aa) was introduced into R751$Tc^R$ plasmid and shown to decrease plasmid stability significantly. Using immunofluorescence we demonstrated that KfrA forms foci co-localizing with R751 plasmid in the *E.coli*. Mutant R751*kfrAΔC* lost the ability to position properly and the plasmid foci became less condensed. Cells carrying defective (and aberrantly located) plasmid R751*kfrAΔC* formed filaments. The role of Upf54.4 and Upf 54.8 in R751 biology was analyzed as well as their ability to interact with KfrA and other plasmid encoded proteins.

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**THE P1 PLASMID IS SEGREGATED TO DAUGHTER CELLS BY A "CAPTURE AND EJECTION" MECHANISM COORDINATED WITH *ESCHERICHIA COLI* CELL DIVISION.** **Yongfang Li and Stuart Austin, Gene Regulation and Chromosome Biology Laboratory, CCR, NCI-Frederick, Frederick, Maryland 21702-1201, USA**

The prophage of bacteriophage P1 encodes a partition system, *par*, which promotes faithful plasmid segregation at low copy number in *Escherichia coli*. The *par* operon produces two *trans*-acting proteins, ParA and ParB which act at a *cis*-acting centromere-like site, *parS*. Analogous systems exist in many low copy number plasmids and homologues of the Par proteins are found in the chromosomes of several bacterial species where they are involved in chromosomal segregation.

ParB binds specifically to the *parS* site in conjunction with the host factor IHF. ParA is an ATPase which interacts with the ParB- *parS* complex. ParB can promote the silencing of genes linked to *parS*. This likely involves ParB loading onto the site and spreading out into the surrounding sequences. We labeled ParB using a GFP-ParB fusion protein. This decorates the DNA in the vicinity of *parS*, with multiple copies of the fluorescent protein and allowed us to follow the fate of the plasmid as it undergoes accurate partition in living cells by time-lapse fluorescence photomicroscopy.

**The dynamic behavior of P1 plasmids during partition.** Figure 1 shows the typical behavior of a P1 mini-plasmid in cells growing and dividing at slow growth on the microscope slide. For a considerable portion of the cell cycle, all plasmid copies are present as a single focus fixed at the cell mid-point. Immediately before cell division, when the central cell septum is close to completion, the focus fragments and is ejected outward along the long axis of the cell. The cell divides and the dispersed foci are free to move and can associate and dissociate.

Figure 1. Typical time-lapse sequence of GFP-ParB foci in cells containing a mini-P1 plasmid. Note that, after partition and cell division, the foci are free to move. One of them splits and then comes back together before finally anchoring at the cell center in preparation for the next cycle.

The GFP-ParB foci presumably mark the positions of plasmid copies. There are generally fewer foci than plasmid copies, so more than one copy can be associated in a focus. The association and dissociation of free foci into fragments presumably reflects association and dissociation of individual plasmid copies. These separations are not productive, because the foci eventually came back together prior to cell division. Eventually a single focus was always formed and became fixed to the new cell center, thus beginning a new cycle. In all cases, ejection was not completed until very late in the cell cycle and the cells divided immediately, trapping one or more focus fragment in each

of the daughter cells. This behavior differs from that previously inferred from statistical analysis of fixed cells where it was proposed that central foci split and rapidly migrated to the cell 1/4 and 3/4 positions at some widely varying point in the cell cycle. Our study shows that the plasmid copies are not transported directly to a particular cell location. Rather, they are dispersed to somewhat random positions and are trapped in each cell half only because cell division follows immediately. Attachment to the new cell center only occurs some time later, presumably in response to synthesis of some cellular attachment site at the new cell center. Initial analysis suggests that the mechanism implied by our observations is compatible with previous studies of fixed cells as long as an assumption is made about the timing of synthesis of new central attachment site in fast growing cells.

**The behavior of plasmids with mutations in the *par* genes.**

Cells containing null mutant plasmids with deletions of ParA and ParB often had no foci. Those cells that did had a single focus, randomly placed within the cell. The focus did not attach to the cell center and cell division left the focus in one cell only.

Fig. 2 shows the time lapse images of wild-type λ-P1:5R in the presence of the dominant negative fusion protein GFP-Δ30ParB. Many of the cells have no focus due to the extreme instability of the plasmid. Here, the focus becomes fixed at the cell center

Figure 2. The cell contains a mini-P1 plasmid mis-segregating under the influence of the dominant negative GFP-Δ30ParB protein. The copies fail to eject from the central binding site and block cell division for some time, giving rise to cell division at an alternative position.

but remains there for a long time without ejection. The cell continues to grow without dividing until it is unusually long. Eventually, the focus is pushed to one side without dispersal, and the cell divides in two places to yield three cells, one of which contains the focus near to the pole. The pattern was similar in other cells starting with central or near-central foci with three or four cells being produced simultaneously. The prolonged attachment of the plasmid appears to interfere directly with some critical step in the septation process at that division site. The elongated cells have the potential to divide again at the 1/4 and 3/4 positions so that divisions for the next generation overlap with the delayed central division.

We speculate that wild-type P1 attachment to the cell center also blocks the cell division. In the wild type case, dispersion of the plasmid copies would allow most cells to divide normally. However, any error resulting in delay to the ejection event would impose a temporary block to cell division, allowing extra time for dispersion to complete. This could increase the fidelity of partition by ensuring that the appropriate events are completed before the cell divides.

# CHARACTERIZATION AND PRACTICAL USE OF THE *mvp* POST SEGREGATION KILLING SYSTEM OF THE LARGE VIRULENCE PLASMID OF *SHIGELLA FLEXNERI*

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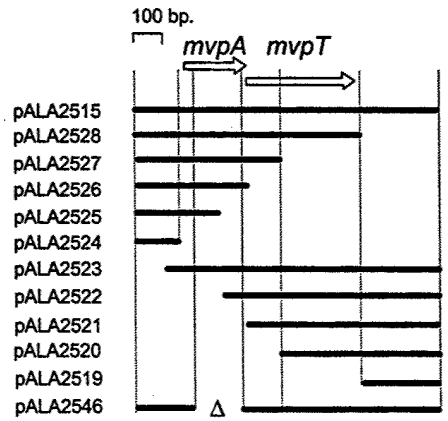
**Plasmid Maintenance Functions of the Large Virulence Plasmid of *Shigella flexneri*.** The ability of *Shigella* and enteroinvasive *Escherichia coli* species to invade epithelial cells and cause enteric disease is dependent on the presence of one of a family of large, low copy number virulence plasmids. The large virulence plasmid pMYSH6000 of *Shigella flexneri* contains a determinant that is highly effective in stabilizing otherwise unstable plasmids in *Escherichia coli* (Radnedge et al., 1997). Expression of the two small contiguous genes, mvpA and mvpT, was shown to be sufficient for stability. Mutations in *mvpT* abolished plasmid stability and plasmids expressing only *mvpT* killed the cells unless *mvpA* was supplied from a separate plasmid or from the host chromosome. When replication of a plasmid carrying the minimal *mvp* region was blocked, growth of the culture stopped after a short lag and virtually all of the surviving cells retained the plasmid. This indicated that the *mvp* system stabilizes by a highly efficient post-segregational killing (PSK) mechanism(Sayeed *et al.*, 2000). Such systems encode a toxin and an unstable antidote. When the plasmid is lost from the cell, the antidote decays but the toxin persists, eventually killing the progeny of the plasmid-free cell. This promotes the maintenance of the plasmid in the growing population. We showed that *mvpT* encodes a cell toxin and *mvpA* an antidote.

**Is stable maintenance of the large virulence plasmid in *Shigella flexneri* dependent on the function of the *mvp* post-segregational killing system?** We introduced a pBR322 plasmid carrying the *mvp* genes into a virulent strain of *Shigella flexneri*. At 37°C, the temperature at which virulence is expressed, the incoming plasmid caused a progressive loss of virulence in the population, as judged by a test for cell-surface virulence factors (the Congo red test). This is consistent with loss of the virulence plasmid. Thus, expression of the *mvpA* gene causes loss of the virulence plasmid implying that the virulence plasmid relies heavily on its *mvp* system for stable maintenance at 37°C. At 30°C, when virulence factors are not expressed, no loss of the virulence plasmid was seen. We have obtained a *Shigella flexneri* strain that carries the virulence plasmid but is avirulent due to a mutation in the *mxi* virulence gene. As the plasmid contains an antibiotic resistance transposon, plasmid




| | % retention of Km$^r$ | |
|---|---|---|
| | 37°C | 30°C |
| pALA2515 | <1 | >98 |
| pALA2528 | <1 | >98 |
| pALA2527 | <1 | >98 |
| pALA2526 | <1 | >98 |
| pALA2525 | >98 | >98 |
| pALA2524 | >98 | >98 |
| pALA2523 | <1 | >98 |
| pALA2522 | >98 | >98 |
| pALA2521 | >98 | >98 |
| pALA2520 | >98 | >98 |
| pALA2519 | >98 | >98 |
| pALA2546 | >98 | >98 |

**Figure 1.** A *Shigella flexneri* strain carrying a kanamycin resistance virulence plasmid plasmid was electroporated with pBR plasmids carrying the indicated portions of the *mvp* region (thick black lines). Retention of kanamycin resistance was determined after approximately 50 generations of unselected growth.

loss can be scored by conventional means. When the cell expressed a supernumerary copy of the *mvp* region at 37°C, this kanamycin resistance marker is rapidly lost. Deletion mapping showed that the critical determinant was the *mvpA* antidote gene (figure 1). We confirmed that the whole plasmid was being lost in these experiments by checking for multiple plasmid sequences by PCR analysis. Thus, the virulence plasmid relies on the *mvp* PSK for its maintenance at 37°C, killing those cells from which the plasmid is lost. When MvpA is expressed from a second plasmid, MvpT is unable to kill the cured cells, and they survive and take over the population.

The virulence plasmid does not rely on *mvp* at 30°C or at lower temperatures that the cells experience in aqueous environments outside of the body. Presumably this is because it encodes efficient replication and partition systems that function well at lower temperatures, so that the *mvp* system is seldom needed. Yet it appears that these functions fail to maintain the plasmid adequately at 37°C, the temperature at which the plasmid virulence factors are expressed during infection of the human colonic mucosa. At this temperature, plasmid maintenance appears to rely heavily on the *mvp* system at the expense of much cell killing. We speculate that efficient cell growth is not needed, and may be even be deleterious during infection. The production of frequent dead cells may actually aid the infection by releasing toxins or by creating a "fire-wall" against attack by the immune system.

**Plasmid Recombineering.** Don Court and co-workers have recently described a powerful method for genetic engineering *in vivo* (Yu *et al.*, 2000). It allows us to manipulate plasmid, phage and chromosomal sequences easily and at will without the severe limitations imposed by the use of restriction endonucleases. We have a special need for a system that can introduce specific mutations into a target gene without leaving a linked marker in the final product. The system should not involve extensive PCR synthesis that introduces accidental secondary mutations. We are working on the scheme that exploits our knowledge of the *Shigella mvp* post-segregational killing system (figure 2). Similar schemes should also allow the construction of hybrid operons in a single step. We are also testing a variant strategy for insertion of point mutations that, starting from a gene duplication, should allow insertion of a mutation anywhere in a gene in a single step.

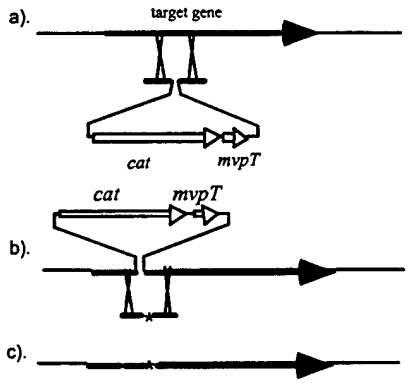



**Figure 2. A recombineering strategy for introduction of point mutations**
a). The plasmid-bourn target gene, present in a strain producing MvpA antitoxin is recombined in vivo with a linear PCR fragment containing the cat-mvpT casette with primer ends homologous to the target sequence thick lines, using λ-red recombination and selecting for chloramphenicc resistance. b). In a second round of recombination, a small oligo spanning the inserted cassette (thick line) and containing the desired point mutation (asterisk) is introduce which restores the target gene with the mutation in place. c) The plasmid is transfered to a strain lacking MvpA. Plasmic retaining the mvpT cassette are killed by the MvpT toxin, thus selecting for the desired product

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**_In Vivo_ Dimerization of the Active Partition Protein IncC of Broad-Host-Range IncP Plasmid RK2.**

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IncP plasmids are broad-host-range, self-transmissible plasmids capable of stable maintenance in a wide range of gram-negative bacteria (8). Studies on the prototypical IncPα plasmid RK2 have provided evidence for the involvement of several different loci in the stable maintenance of the plasmid. These include loci for replication control, multimer resolution, plasmid addiction and active partition, as well as host-specific stability loci whose mechanisms of action have yet to be determined (7, 8, 12). The IncP plasmid active partition system is similar to, but distinct from, the prototypical plasmid partition systems on the P1 and F plasmids. The partition components include the *incC* and *korB* genes and the DNA site ($O_B$) to which the *korB* gene product binds (2, 6, 7, 9). The *incC* gene encodes two polypeptides: a 38.1 kD IncC1 protein and a shorter 27.5 kD IncC2 protein (8). Both IncC1 and IncC2 are members of the ParA family of partition ATPases found in many bacterial and archaeal species (4, 7). KorB functions both as a global transcriptional repressor of six different RK2 operons and as a DNA-binding protein involved in partition (8). It is a homolog of the ParB family of partition proteins that promote partition in conjunction with their ParA counterparts via a ParB-specific DNA site on the target plasmid (4). In the case of RK2, IncC and KorB have been shown to interact *in vivo* and *in vitro* (9). Partition is believed to occur by the pairing of plasmid siblings at or near the mid-cell and their subsequent separation towards the poles of the cell prior to cell division (1). The ParA and ParB homologs of several partition systems have been shown to form nucleoprotein complexes at their cognate DNA sites (2, 11). These complexes are believed to mediate plasmid pairing and separation. Evidence for the involvement of the ParB homolog in plasmid pairing has emerged from work on the R1 and P1 plasmid partition systems (3, 5). What role the ParA homolog plays is less clear, although the ATPase activity of the ParA protein is postulated to play a role in the separation of paired plasmids. We have shown that overexpression of IncC causes the rapid loss of RK2, a process that probably reflects plasmid aggregation due to inter-molecular pairing between the 12 $O_B$ sites on each RK2 molecule (9). IncC could promote plasmid pairing via IncC-IncC interactions or by enhancing KorB-KorB interactions. We first tested if IncC2 is capable of dimerization in a bacterial one-hybrid assay. In this assay, developed by James C. Hu of the Texas A&M University, a fusion of IncC2 to the DNA-binding domain of the phage lambda cI repressor is expressed in trans to a lambda $P_R$-driven *lacZ* reporter. Full length lambda cI binds as a dimer to its operator in the lambda $P_R$ promoter and represses transcription of *lacZ*. Removal of the lambda cI C-terminal dimerization domain results in a DNA-binding protein that can bind its operator in the $P_R$ promoter but is unable to repress transcription. Expression of a cI DNA binding domain-IncC2 fusion protein restored repression of *lacZ* and provided immunity to lambda superinfection, indicating that IncC2 is able to provide dimerization activity to the fusion protein. We also constructed an IncC2-T7•tag fusion that is able to fully

complement an *incC* deletion mutant of the IncPα plasmid R995 (10). At physiological levels required for complementation, IncC2-T7 forms a species *in vivo* that migrates as a dimer in SDS-PAGE, as evidenced by *in vivo* formaldehyde crosslinking experiments. These data suggest that IncC-IncC interactions may play a role in RK2 partition, perhaps in the pairing of KorB-$O_B$ nucleoprotein complexes. Work to determine the location of the dimerization domain and the precise role of IncC dimerization in partition is currently under way.

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Dissection of the mini-replicon of IncP-9 plasmid pM3

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IncP-9 plasmids are generally medium to large self-transmissible plasmids that are stable in *Pseudomonas putida* and closely related species. They are able to transfer to many other Gram-negative bacteria but are less stably inherited – for example, being temperature sensitive for maintenance in *Escherichia coli*. We have recently sequenced pWW0, the archetypal IncP-9 TOL plasmid (1). The mini-replicon from another IncP-9 plasmid, pM3, was also sequenced (2) and shows high sequence identity to the equivalent region of pWW0. The proposed maintenance functions of pMT2 are in a single block and consist of: *parA parB*, *korA*, *tolA*, *res oriV* and *rep*, all orfs being transcribed from left to right from putative promoters upstream of *parA*, *res* and *rep*. Two strong incompatibility determinants were mapped to the *parA* promoter region and the *oriV-rep* region. Inactivation of *res* had no detectable effect on maintenance, whereas knock-outs in the *par* region had a severe negative effect on maintenance. On the other hand PCR amplification of *oriV* and *rep* gave a segment that could replicate, demonstrating that the basic replication machinery must be encoded by these two determinants. *E.coli* RNA polymerase binding studies and reverse transcriptase primer extension mapping of the mRNA start identified the promoter for *rep* just upstream of *rep* and within the putative *oriV* region. Deletion analysis and reporter gene fusions confirmed the location of this promoter and showed that it is autoregulated by Rep. Rep was purified and binding studied by gel retardation and footprinting, demonstrating binding to a series of repeats in the *oriV* region and overlapping the *rep* promoter region, there being a hierarchy of binding strengths starting at the proposed autoregulatory region and becoming progressively weaker towards the other end of *oriV*, away from *rep*. Two putative DnaA binding sites were identified from sequence analysis. Studies with DnaA from *E.coli*, *P. putida* and *P. aeruginosa* showed little evidence of significant binding with any of these proteins alone. However, when Rep was present close to its $K_{app}$, addition of any of the DnaAs caused an additional complex to form. This suggested that Rep can recruit DnaA to *oriV*. There also appeared to be interaction between DnaA and RNAP and this fits with observations that full *rep* promoter activity is dependent on a region containing one of the two putative DnaA boxes. Therefore, *rep* transcription may be dependent on or stimulated by the presence of DnaA. This compact mini-replicon may therefore represent an excellent system to look at the interaction of host and plasmid functions needed for maintenance.

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**The highly conserved TldD and TldE proteins of *Escherichia coli* are involved in Microcin B17 processing and in CcdA degradation**

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Microcin B17 (MccB17) is a peptide antibiotic produced by *Escherichia coli* strains carrying the pMccB17 plasmid. MccB17 is synthesized as a precursor containing an amino-terminal leader peptide that is cleaved during maturation. Maturation requires the product of the chromosomal *tldE* (*pmbA*) gene. Mature microcin is exported across the cytoplasmic membrane by a dedicated ABC transporter. In sensitive cells, MccB17 targets the essential topoisomerase II DNA-gyrase. Independently, *tldE* as well as *tldD* mutants were isolated as being resistant to CcdB, another natural poison of gyrase encoded by the *ccd* poison/antidote system of plasmid F. This led to the idea that TldD and TldE could regulate gyrase function. We present in vivo evidence supporting the hypothesis that TldD and TldE have proteolytic activity. We show that in bacterial mutants devoid of either TldD or TldE activities, the MccB17 precursor accumulates and is not exported. Similarly, in the *ccd* system, we found that TldD and TldE are involved in the CcdA and CcdA41 antidote degradation rather than being involved in the CcdB-resistance mechanism. Interestingly, sequence database comparisons revealed that these two proteins have homologues in eubacteria and archaebacteria, suggesting a broader physiological role.

# *TRANSFER*

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**84** Ti plasmid conjugal donors: Turning them on, turning them off. Stephen K. Farrand, Yinping Qin, and Shengchang Su

**86** Repression and epidemic spread: Two levels of *traJ* regulation in the F plasmid. Laura S. Frost

**87** Bacterial conjugation: A two-step mechanism for DNA transport. Matxalen Llosa, F. Xavier Gomis-Rüth, Miquel Coll and Fernando de la Cruz

**88** Bacterial conjugative transfer: Visualization of successful mating pairs and plasmid establishment in live *Escherichia coli*. Trevor D. Lawley and Diane E. Taylor

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**97** Features of the pMV158-encoded protein MobM and the plasmid origin of transfer, *oriT*. Carmen de Antonio, María Eugenia Farías[1], Concepción Nieto,

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**115** Molecular analysis of the sequence-specificity differences between R100 and F factor TraI nuclease domains. Matthew J. Harley and Joel F. Schildbach

**116** Peptidoglycan hydrolysis by P19 and related proteins from type III and type IV secretion systems. Doris Zahrl, Barbara Zavecz, Gernot Zarfel, Christoph Ruckenstuhl and Günther Koraimann

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William R. Will, Karen G. Anthony, and Laura S. Frost

# BIOGENESIS OF THE T-DNA TRANSFER SYSTEM OF *AGROBACTERIUM TUMEFACIENS*

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The T-DNA transfer system of *Agrobacterium tumefaciens* delivers DNA and proteins to plant cells by a contact-dependent mechanism reminiscent of conjugation. Recent studies have determined that systems related to the T-DNA transfer system are used by many bacteria for conjugal DNA transfer and by pathogens for delivery of effector proteins to mammalian cells during the course of infection. These systems are now collectively referred to as the type IV secretion family.

This laboratory is characterizing the assembly pathway, structure, and function of the T-DNA transfer system. The 11 VirB proteins and the VirD4 protein coordinate their activities to assemble this system. Recent studies implicate VirD4 as a coupling protein and possible translocase responsible for delivery of secretion substrates across the inner membrane. The VirB proteins elaborate the T pilus for establishment of donor-target cell contacts. However, genetic studies indicate that the VirB proteins also participate in substrate translocation.

The VirB11 ATPase is a member of a large family of NTP-binding proteins whose functions generally are required for assembly of supramolecular surface organelles or secretion machines. Based on electron microscopy studies of several VirB11 homologs, including TrbB of plasmid RP4, TrwD of R388, and HP0525 from *Helicobacter pylori*, and the HP0525 crystal structure, the members of this ATPase superfamily are thought to assemble as homohexameric, double ring structure whereby the ring encoded by the N-terminal half of the protein associates with the cytoplasmic membrane and the ring encoded by the C-terminal half is exposed to the cytoplasm. This structure is postulated to undergo ATP-dependent conformational changes to drive machine assembly and/or substrate translocation.

To characterize the role of VirB11 in assembly or function of the T-DNA transfer system, we examined the effects of *virB11* mutations on VirB protein accumulation, T-pilus production, and substrate translocation. Strains synthesizing VirB11 derivatives with mutations in the nucleoside triphosphate binding site (Walker A motif) accumulated wild-type levels of VirB proteins but failed to produce the T-pilus or export substrates at detectable levels, establishing the importance of nucleoside triphosphate binding or hydrolysis for T-pilus biogenesis. Analyses of strains expressing *virB11* dominant alleles in general showed that T-pilus production is correlated with substrate translocation. Notably, strains expressing dominant alleles previously designated class II (dominant and nonfunctional) neither transferred T-DNA nor elaborated detectable levels of the T-pilus. By contrast, strains expressing most dominant alleles designated class III (dominant and

functional) efficiently translocated T-DNA and synthesized abundant levels of T pilus. We did, however, identify four types of *virB11* mutations or strain genotypes that selectively disrupted substrate translocation or T-pilus production: (i) *virB11/virB11** merodiploid strains expressing all class II and III dominant alleles were strongly suppressed for T-DNA translocation but efficiently mobilized an IncQ plasmid to agrobacterial recipients and also elaborated abundant levels of T pilus; (ii) strains synthesizing two class III mutant proteins, VirB11, V258G and VirB11.I265T, efficiently transferred both DNA substrates but produced low and undetectable levels of T pilus, respectively; (iii) a strain synthesizing the class II mutant protein VirB11.I103T/M301L efficiently exported VirE2 but produced undetectable levels of T pilus; (iv) strains synthesizing three VirB11 derivatives with a four-residue (HMVD) insertion (L75.i4, C168.i4, and L302.i4) neither transferred T-DNA nor produced detectable levels of T pilus but efficiently transferred VirE2 to plants and the IncQ plasmid to agrobacterial recipient cells. Together, our findings support a model in which the VirB11 ATPase contributes at two levels to type IV secretion, T-pilus morphogenesis, and substrate selection. Furthermore, the contributions of VirB11 to machine assembly and substrate transfer can be uncoupled by mutagenesis.

Other work in this laboratory has focused on defining steps in the biogenesis of this secretion system. We have determined that VirB7, a small outer membrane lipoprotein, interacts with VirB9 via formation of an intermolecular disulfide crosslink, and that assembly of this heterodimer at the outer membrane is essential for recruitment and stabilization of several inner membrane VirB proteins including VirB4, VirB10, and VirB11. The polytopic inner membrane protein VirB6 also participates in the assembly process. Results of studies utilizing immunoprecipitation and GST pull-down assays to characterize the requirements for assembly of VirB protein subcomplexes will be summarized.

Finally, we have mutagenized the inner membrane VirB6 and outer membrane VirB9 proteins by insertion of 2 or 4 residues along the lengths of the proteins. Phenotypic studies led to isolation of additional "uncoupling" mutations in both proteins. Based on these findings, we propose that a core structure composed of the outer membrane VirB7-VirB9 heteromultimer and the bitopic inner membrane protein VirB10 functions as a platform for the further assembly of the T pilus and a structure that mediates substrate translocation minimally across the outer membrane. In wild-type cells, these two structures – the pilus and the translocation channel – might be physically connected. However, the isolation of uncoupling mutations in VirB6, VirB9, and VirB11 supports the notion that the two structures can also be assembled independently of each other.

**Conservation of a C2H2-zinc finger-containing regulator of virulence and oncogenes of the Ti plasmid**

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Two-component regulatory systems responsive to environmental stimuli are well documented among many distinct bacteria, including *Agrobacterium tumefaciens*, in which VirA and VirG are representatives that positively regulate virulence (*vir*) genes on the Ti plasmid. A less known negative regulation system of *vir* and oncogenes is controlled by Ros, a 15.5 kDa protein which is encoded by the chromosomal gene, *ros*. The *ros* gene was discovered through genetic screening for up-regulated mutants of the *virC* and *virD* operons. The *ros* gene has been genetically mapped and is located between *his-34* and *trp-19*. The Ros protein contains a novel C2H2-zinc finger that was thought to only exist in mammalian and human species. Ros contains one mole of $Zn^{2+}$, whose retention in Ros depends on the Cys and His residues within the finger. Also, the loss of either residue, including Arg-125 near the carboxyl terminus causes derepression of T-DNA processing (in the absence of induction) and the inability to bind the *ros* box upstream of the *virC* and *virD* operons. Ros also represses the expression of the T-DNA gene, *ipt*, which encodes isopentenyl transferase leading to the formation of cytokinin. Mutation of *ros* causes the formation of cytokinin, T-DNA intermediates and loss of acidic succinoglycan production in *A. tumefaciens*. Comparative sequence analyses revealed the presence of *ros* in *A. radiobacter*, *Sinorhizobium meliloti*, *Rhizobium etli*, *Rhizobium* sp. NRG234 and *Sphingomonas aromaticivorans*. Distance tree analysis has indicated that the Japanese puffer fish, *Fugu rubripes*, rather than plant species is the nearest species containing a potential *ros*-like zinc finger. We therefore hypothesized that *ros* may have originated from a marine environment. Hence, we examined microbes and lower eukaryotes derived from marine habitats for the presence of *ros* in their genomes and plasmids. Several bacteria derived from the marine sediments were grown on sea water containing media. These bacteria were found to contain *ros* as evidenced by Southern blot hybridizations using bona fide *ros* as a molecular probe. The results helped support the above hypothesis and have redirected our focus towards marine bacteria as the possible origin of *Agrobacterium* species.

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# Ti Plasmid Conjugal Donors: Turning Them On, Turning Them Off

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Conjugal transfer of Ti plasmids from donor *Agrobacterium tumefaciens* strains to agrobacterial recipients in natural environments is a tightly regulated process. The three Ti plasmid transfer operons, *traAFB*, *traCDG*, and *traI-trb* normally are expressed only when the donors are colonizing the crown gall tumors they induced on their susceptible plant hosts. The regulatory system responds to two signals in a hierarchical manner. The first, the conjugal opines, are small carbon compounds produced specifically and uniquely by the plant tumors. The opines serve to induce expression of the primary *tra* regulon activator, TraR, which is a member of the LuxR family of quorum-sensing transcription factors. TraR, in turn responds to the second signal, *Agrobacterium* Autoinducer (AAI), an acylated homoserine lactone that is produced by the donor bacteria themselves. AAI, which partitions out of and back into the cells in a stochastic fashion accumulates in the environment as the bacterial population increases in size. When the quormone reaches a critical concentration signifying the desired donor population size it drives the dimerization of TraR to its active form. Thus, the conjugal opine signals to the Ti plasmid that the environment is favorable for its transfer to other bacteria while the quorum-sensing system serves to delay transfer until the donor population has reached an effective size.

The activity of TraR is modulated by a small antiactivator protein, TraM, also coded for my the Ti plasmid. This protein binds to the carboxyl-terminal end of monomer and dimer forms of TraR and prevents the functional activator from binding to its DNA target site. TraM is essential for the quorum-sensing phenomenon; otherwise wild-type Ti plasmids with mutations in *traM* transfer constitutively even in the absence of induction by the opines. These observations indicate that in the absence of the conjugal opine *traR* is expressed at a basal level that is sufficient to activate transfer. We propose that one role of TraM is to prevent premature conjugation attendant to this low level of expression of the activator gene. In addition, Ti plasmids with mutations in both *traM* and the opine regulatory system are hyperconjugal, suggesting that the antiactivator also serves to govern the level of expression of the *tra* regulon once induced by the conjugal opine. Thus, TraM is important in regulating the initiation and level of expression of the Ti plasmid conjugal transfer system.

Although we understand how Ti plasmid transfer is induced, little attention has been given to how this highly regulated system is deactivated when the conjugal opines become limiting. *A. tumefaciens* produces an enzyme that hydrolyzes the acyl-homoserine lactone ring, thus inactivating the quorum signal (Zhang, H.-B., Wang, L.-H., and Zhang, L.-H. 2002. Proc. Natl. Acad. Sci. USA. 99:4638-4643). This activity, which is strongly induced when the cells enter into stationary phase, effectively lowers the concentration of the quormone to levels insufficient to activate TraR. However, in a reporter system containing only *traR* and a *traG::lacZ* fusion the dimer form of TraR

retains activity long after removal of the quormone suggesting that the active protein is very stable. Our recent studies using Western analysis have shown that in the absence of TraM, active TraR is indeed very stable within the cells. However, when TraM is co-expressed, TraR, even in its active dimer form, is rapidly degraded. Screens using appropriate mutants of *E. coli* indicate that Lon protease is largely if not entirely responsible for the TraM-dependent proteolysis of TraR; in two independent *lon* mutants the activator is not subject to enhanced degradation even when TraM is co-expressed. Expression of the *lon* homolog from *A. tumefaciens* complemented this defect in *E. coli*. However, in mutants defective in ClpX, ClpP, ClpA and ClpQ TraR continues to be rapidly degraded in a TraM-dependent manner. We conclude that TraM serves a third function and that is to deactivate the conjugation system by targeting TraR for degradation when the environmental conditions no longer are favorable for transfer of the Ti plasmid.

**REPRESSION AND EPIDEMIC SPREAD: TWO LEVELS OF *TRAJ* REGULATION IN THE F PLASMID.** Laura S. Frost Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

F-like plasmids encode a transfer region encoding two monocistronic operons for *traM* and *traJ* and the multicistronic *tra* operon (31.5 kb or more). The positive activator of *tra* operon expression, TraJ, acts at the Py promoter to express *traY* the first gene in the *tra* operon. TraY, in turn, further activates Py and the *traM* promoters which are autoregulated by the TraM protein that binds in a cooperative fashion to its three binding sites upstream of the *traM* gene. Overlaying this control circuit is the fertility inhibition system comprised of an antisense RNA FinP and the FinO protein that both stabilizes FinP and promotes duplex formation with the untranslated leader region of the *traJ* mRNA, downregulating TraJ translation and *tra* operon expression. F is naturally derepressed by an IS*3* insertion in *finO* and can be easily repressed by the addition of exogeneous FinO from a compatible plasmid. While the control elements described above are readily apparent in exponentially growing cells, these plasmids also have a complex array of control elements that tweak the system in response to changes in their environment. We have concentrated on three such conditions including the loss of mating ability in stationary phase, the response to extracytoplasmic stress and the presence of a large excess of recipient cells over donor cells.

Maximal levels of conjugation are seen in early exponential phase in F+ cells grown in rich media under aerobic conditions. The conjugative ability falls dramatically as cells enter mid-exponential phase although the levels of TraJ are maintained. Instead, the key regulatory element, at least at the transcriptional level, appears to be TraY which rises and falls during a period in growth that corresponds to maximum mating ability. The control of *traY* at the Py promoter appears to be influenced by the histone-like proteins Fis and IHF which cooperate to repress the Py promoter.

Extracytoplasmic stress is monitored, in part, by the Cpx regulon, which prevents the accumulation of TraJ in a post-transcriptional manner. This suggests that CpxA and R regulate a new pathway that specifically degrades TraJ and prevents conjugation at inopportune times. However, when the opportunity presents itself, repressed F plasmids adopt a mode called "High Frequency of Transmission" or HFT, and mate over several generations at derepressed levels to ensure "epidemic spread" of the plasmid throughout the recipient cell population. This epidemic spread is the result of transcription of *traM* at full throttle which downregulates the *traJ* gene. Epidemic spread can be curtailed by supplying *traM* transcripts in the recipient cell thereby supplying TraM which represses the Pm promoters on the incoming F DNA.

A picture is emerging of the optimum conditions for conjugative transfer by the IncF complex of plasmids. They prefer warm, rich liquid media where their mission is to introduce the plasmid to all available recipient cells. They downregulate plasmid transfer at the slightest sign of stress or metabolic decline by involving participation by host regulatory factors. Consequently, the emergence of multiply antibiotic-resistant plasmids of the IncF group in warm, wet climates is perfectly consistent with their modus operandi.

# BACTERIAL CONJUGATION: A TWO-STEP MECHANISM FOR DNA TRANSPORT

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Bacterial conjugation is a promiscuous DNA transport mechanism. Conjugative plasmids transfer themselves among most bacteria, thus being one of the main causal agents of the spread of antibiotic resistance among pathogenic bacteria. In addition, DNA can be transferred conjugatively into even eukaryotic hosts. In this talk we will address several basic questions regarding the DNA transfer mechanism. Our model is principally based on the 3D structure and biochemical activities of TrwB, the coupling protein of plasmid R388.

Conjugation can be visualized as a DNA rolling-circle replication (RCR) system plugged into a Type IV secretion system (T4SS). Conjugative DNA processing by relaxases and accessory proteins is reminiscent of ssDNA phage RCR. The set of proteins forming the transport structure are organized as a T4SS. These are protein transporters widely implicated in bacterial pathogenic mechanisms. The assemblage "replication + secretion" suggests how the mechanism would work on the DNA substrate and across the bacterial membranes. We postulate that the T4SS is responsible for transport of the pilot protein (the relaxase) to the recipient. The DNA that is covalently linked to it is initially transported in a passive manner, trailing on the relaxase. We propose that the pilus appendage could work as a catapult or a syringe, thrusting the substrate proteins to cross one or several membrane barriers into the recipient cytoplasm. This is the first step in conjugation. The second step is the active pumping of the DNA to the recipient, using the already available T4SS transport conduit. It is proposed that this second step is catalyzed solely by the coupling proteins. Thus coupling proteins could fulfil two basic roles in conjugation: coupling the two main components of the machinery (RCR and T4SS) during the first step in conjugation, and actively mediating DNA transport during the second step.

Arguments in favour or against each of the hypothesis in the model will be discussed.

**BACTERIAL CONJUGATIVE TRANSFER: VISUALIZATION OF SUCCESSFUL MATING PAIRS AND PLASMID ESTABLISHMENT IN LIVE *ESCHERICHIA COLI*.**

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We utilized the LacO/GFP-LacI system to fluorescently label and visualize the IncPβ plasmid R751 during conjugative transfer between live donor and recipient bacteria (1). Comparisons of R751 during conjugative and non-conjugative conditions has allowed us to identify key localizations and movements associated with the initiation of conjugative transfer in the donor and the establishment of R751 in the recipient. A survey of successful mating pairs demonstrates that close physical contact between donor and recipient bacteria is required for DNA transfer and that regions of intimate contact can occur at any location on the donor or recipient cell membrane. The transferred DNA is positioned at the characteristic center or quarter-cell position after being converted to a double-stranded molecule in the recipient cell. Initial duplication of plasmids often results in an asymmetric distribution of plasmid foci. Symmetric localization (either at center or at 1/4 and 3/4 cell lengths) occurs only after a significant lag, presumably reflecting the time required to synthesize the plasmid-encoded partitioning proteins.

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# INTERACTION OF THE RELAXOSOMAL PROTEIN $TRAM_{R1}$ WITH THE INNER MEMBRANE PROTEIN $TRAD_{R1}$: COUPLING THE DNA SUBSTRATE TO THE TRANSPORTER.

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The TraM protein of the IncFII antibiotic resistance plasmid R1 has several roles in plasmid DNA transfer. As a plasmid specific DNA binding protein it binds in a tetrameric form to multiple binding sites in the plasmid R1 *oriT* (6, 7), thereby participating in relaxosome formation. It enables efficient cleavage by the TraI protein at the R1 *nic* site *in vivo* (2), and it is also involved in maintaining a complex regulatory circuit in the control of plasmid R1 transfer gene expression (3). It was recently shown in the case of the closely related F plasmid that $TraM_F$ interacts with the inner membrane protein $TraD_F$ (1); the results of genetic studies indicated a specific requirement for C-terminal sequences of TraD (4). Because of these findings and since TraD belongs to the TraG-like proteins which are believed to couple the substrate to the transporter, we set up several *in vitro* experiments to investigate this interaction in the R1 system. Furthermore, we wished to determine whether this interaction was plasmid specific or independent of the plasmid system. In overlay assays, in which we used different purified $TraM_{R1}$ and $TraD_{R1}$ proteins variants, we observed that these proteins, as expected, also interact in the R1 plasmid conjugation system. The interaction required the presence of the C-terminal residues of the TraD protein, the N-terminal membrane spanning domain was not necessary. For the TraM protein it was not possible to map the interaction domain either to the C- or N-terminal half of the protein. ELISA with the purified protein variants showed that the proteins interact with each other specifically in the nanomolar range. Co-immuno precipitation experiments demonstrated that $TraM_{R1}$ also interacts with an N-terminally His tagged $TraD_F$ protein showing that this interaction is not plasmid specific. A C-terminally His tagged $TraD_F$ protein did not interact with $TraM_{R1}$, a finding that is supported by *in vivo* data (5). In this protein the His tag probably blocked this interaction, again demonstrating that the interaction domain contains the C-terminal amino acid residues of TraD.

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# SOME NEW CONNECTIONS BETWEEN REPLICATION AND CONJUGAL DNA TRANSFER

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The three Mob proteins of plasmid R1162, the relaxase and two small accessory proteins, assemble at *oriT* to form the relaxosome. In addition to being essential for conjugal transfer, these proteins regulate transcription from the three promoters adjacent to *oriT* (1), and in this way regulate their own expression as well as that of the plasmid *rep* genes. Derepression of these genes due to a defective relaxosome causes a two- or three-fold increase in copy-number. Mutations in *mobB*, which encodes one of the accessory proteins, destabilize the relaxosome, and this causes gene overexpression with a toxic effect on the cells (2). Two of these mutations could be suppressed by additional copies of *oriT* in trans, cloned in the vector pBR322. One interpretation of this suppression is that the additional copies of *oriT* are stabilizing partially-defective complexes by means of intermolecular "handcuffing". We analyzed plasmid dimer molecules by two-dimensional gels (3) to demonstrate molecular handcuffing at *oriT* in vivo.

MobA, the *oriT* relaxase encoded by R1162, contains two domains having different functions in the relaxosome. An amino-terminal part of the protein, which has been mapped by both genetic and biochemical methods (4), consists of about 188 amino acids and contains the DNA strand-cleaving and rejoining activity. Adjacent to this is a region required for stabilization of the relaxosome by MobB. We have shown the functional independence of this second domain, and its interaction with MobB, by replacing it with the comparable region of the related relaxase from pSC101. In this case, the relaxosome is stabilized only by the cognate pSC101 MobB analog, MobX, while the relaxase still recognizes the R1162 *oriT* (5). Deletion of this domain also results in dimer molecules no longer exhibiting intramolecular handcuffing.

A donor cell can transfer more than one copy of R1162 DNA into a recipient cell when the IncP-1 plasmid R751 is the mobilizing vector (6). Do these multiple rounds of transfer originate from the same molecule, tethered to the conjugal pore in the donor cell, or from different molecules (perhaps jointly brought to the conjugal pore by handcuffing)? We have been examining conjugal transfer under conditions where vegetative replication of the transferring plasmid in donor cells is strictly prevented. An R1162 derivative deleted for the *rep* and *mob* genes was electroporated into donor cells containing the Mob gene products. The newly transformed cells were then immediately mated with recipient cells providing the Rep proteins (7). The plasmid DNA was constructed so that it contained a single base mismatch. To inhibit mismatch repair, DNA was hypermethylated prior to electroporation (8). The base-pair mismatch results in an NheI restriction site in the strand transferred during conjugation, and an FspI site at the same location in the other strand. Thus, the complement to the FspI strand must be synthesized before this restriction site will appear in the DNA transferred to a recipient.

When the test plasmid was electroporated into the Rep+ donor and the cells immediately mated, NheI+ and FspI+ plasmids were present in equal numbers in the pooled population of transconjugant cells. In addition, about 50% of individual transconjugant colonies contained both NheI+ and FspI+ plasmid molecules, in about equal amounts. Since there were many more potential recipient cells than donor cells in the mating mixture, the possibility is small that a recipient cell formed a mating pair with more than one donor cell. This confirms that there is frequently transfer of more than one plasmid molecule from a single donor cell.

To determine whether multiple rounds of transfer depend on vegetative replication to provide copies of the plasmid, we next asked about the distribution of the restriction sites when the donor was Rep-. In this case, there were more NheI+ plasmids than FspI+ plasmids in the entire population of transconjugants. However, a small proportion of individual colonies did contain both NheI+ and FspI+ plasmids. In contrast to the Rep+ case, the amount of DNA for each was not equal, but instead there were many more NheI+ plasmids in the colony. Either strand replacement synthesis in the donor is inherently inefficient, or significant time elapses before the molecule is again active in a round of transfer. During this time, the conjugal pore might close, or the first molecule might have replicated sufficiently so that establishment of the second molecule is inhibited by incompatibility. We repeated this experiment with plasmid molecules having a different base-pair mismatch, resulting in a mutation in the *oriV* of the strand transferred first. This mutation affects strand separation at the AT-rich region within *oriV* (9), and probably loading of the plasmid-specific helicase. It is not likely to prevent transfer or single-strand replacement synthesis, but would prevent both subsequent vegetative replication in the recipient and thus entry exclusion by incompatibility. Plasmids with this mutation had a much higher frequency of second strand transfer from the Rep- donors. It appears that a single plasmid molecule can participate in more than one round of transfer, even in the absence of ongoing vegetative replication. However, multiple rounds of transfer do not seem to involve the rapid spooling of plasmid copies into a recipient from a single molecule in the donor. Instead, there is significant "eclipse period" after a round of transfer, when the plasmid is refractory to mobilization.


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# THE MULTIPLE ROLES OF TRAI IN CONJUGATIVE TRANSFER OF F PLASMID DNA

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TraIp (also called DNA helicase I) is an *Escherichia coli* F plasmid-encoded protein required for conjugative DNA transfer. The purified protein catalyzes a sequence-specific DNA transesterase reaction that provides the site- and strand-specific nick required to initiate DNA strand transfer, and a 5' to 3' DNA helicase reaction that unwinds the F plasmid to provide the single-stranded DNA that is transferred from donor to recipient. Sequence comparisons with other transesterases and helicases suggest that these activities reside in the amino- and carboxyl-terminal regions of TraIp, respectively. Computer-assisted secondary structure probability analysis identified a potential inter-domain region spanning amino acid residues 305-309. Proteins encoded by segments of *traI*, whose N- or C-termini either flanked or coincided with this region, were purified and assessed for catalytic activity. Amino acids 1-306 contain the transesterase activity of the protein. A smaller protein fragment containing residues 1-235, and identified as a stable domain in partial proteolysis studies, lacks transesterase activity and fails to bind a single-stranded DNA substrate containing the F plasmid origin of transfer (*oriT*). Thus, the region of TraIp extending from residue 235 to residue 306 is necessary for a stable interaction of the transesterase with *oriT* DNA. The tyrosines involved in the transesterase reaction are located at the amino terminal end of the protein. Molecular genetic and biochemical experiments suggest a novel relay mechanism for transesterification. Amino acids 309-1504 harbor the helicase activity of TraIp. Surprisingly, residue 309 defines the amino terminal end of the helicase domain; removal of an additional 40 amino acids abolished helicase activity while increasing the apparent $K_d$ for binding DNA slightly. This is remarkable since the first helicase-associated motif (helicase motif I) is located approximately 650 amino acids further toward the C-terminal end of the protein. The region of the protein from position 306 to position 990 shares no homology with other helicases and has no known role. Genetic complementation experiments using plasmids designed to express either the transesterase domain, the helicase domain or both domains as separate polypeptides have shown that both domains of the protein are required for conjugative DNA transfer. Moreover, they must be part of a single polypeptide chain. In addition, we have made a point mutation in TraIp (K998M) that alters the essential lysine in helicase motif I to methionine. This protein, which catalyzes a transesterase reaction but lacks both helicase and ATPase activity, fails to support conjugative transfer of the F plasmid. Thus, transfer-associated unwinding of the F plasmid requires the TraIp helicase activity. We have also defined a third functional domain of helicase I represented by the C-terminal 252 amino acids of the protein (i.e. residues 1504-1756). Purified TraIp lacking the C-terminal 252 amino acids retains essentially wild-type helicase and transesterase activity but fails to complement the loss of helicase I in genetic complementation assays. We speculate that the C-terminal end of TraIp is required either for formation of an active relaxosome or for an interaction with membrane-associated components at the next step in the DNA transfer pathway.

The F plasmid conjugative initiation complex (the relaxosome) involves the assembly at *oriT* of at least two proteins in addition to TraIp, IHF and TraYp. IHF and TraYp, both site-specific DNA binding proteins, apparently modulate the chromatin architecture at *oriT* to facilitate binding of TraIp. *In vitro* studies have shown the *oriT*-dependent transesterase activity of native TraIp is stimulated in the presence of both IHF and TraYp, but not if one or the other is omitted. Using purified segments of TraIp, we have studied their contributions to the formation of a functional initiation complex at *oriT*. We found IHF to be a potent inhibitor of *oriT* nicking by the functional N-terminal transesterase segment of TraIp, N306. Addition of TraYp alone did not relieve the inhibition. In the absence of TraYp inhibition was diminished somewhat in the presence of either one of two large C-terminal segments of TraIp. One of these was a functional helicase (309C), while the other was catalytically inert (348C). Stimulation of N306 transesterase activity, comparable to that of native TraIp, was only observed in the presence of IHF, TraYp, and 309C. 348C could not functionally substitute for 309C in this respect. The observation that IHF conditionally inhibits and stimulates functional initiation at *oriT* suggested a more complex role for this protein than had previously been supposed. Sequence analysis of the *nic*-proximal region of *oriT* revealed the potential for two mutually exclusive and inverted IHF binding sites distinct from the previously assigned IHF binding site. These results are considered in the context of previously published reports regarding protein-DNA interactions at *oriT*, as well as IHF in general, and the recent IHF-DNA crystal structure.

# MAPPING THE ACTIVE SITE OF THE F FACTOR TRAI RELAXASE ACTIVITY

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F TraI and other relaxases of conjugative plasmids cleave one plasmid DNA strand prior to transfer of the cut strand from donor to recipient. Although relaxases were known to preferentially recognize the *oriT* of the plasmid that encodes them, the level of specificity of single-stranded DNA (ssDNA) recognition by relaxases had not been closely examined until recently. We previously demonstrated that F TraI recognizes a single-stranded region of F *oriT* with a subnanomolar dissociation constant and remarkable sequence-specificity. These experiments were performed with a fragment of F TraI, dubbed "TraI36," that retained relaxase activity. $Mg^{2+}$ is an absolute requirement of the relaxase ssDNA cleavage reaction, and by excluding it from the binding buffer we were able to follow reversible binding reactions in solution. Binding was detected by changes in fluorescence emission intensity and anisotropy of a carboxytetramethylrhodamine-labeled oligonucleotide.

More recently we have sought to determine how F TraI recognizes and cleaves ssDNA. Using limited trypsin digestion, we have found that loss of the C-terminal 10 kDa of TraI36 renders the protein incapable of high affinity ssDNA binding. We have found that substitution of Phe for either Tyr16 or Tyr17 renders TraI36 incapable of oligonucleotide cleavage. By substituting Ala for various amino acids in TraI36 that are conserved among related relaxases, we have identified a number of residues that are involved in ssDNA recognition. One of these, R150, is involved in recognition of DNA located 3′ to *nic*, the site of oligonucleotide cleavage. Finally, we are generating F/R100 chimeras of TraI36. F and R100 TraI share 91% sequence identity over their first 330 amino acids, yet they clearly discriminate between their *oriT* binding sequences. We are swapping R100 sequences into the F TraI36 background to identify the amino acids responsible for the specificity difference. The great majority of substitutions cause no change in specificity, suggesting that a few amino acid changes are responsible for the specificity difference. By combining results from these various approaches, we are beginning to map out the F TraI relaxase binding site. (This presentation is based on work supported by the National Science Foundation under Grant MCB-9733655.)

Analysis of protein-protein interactions between the F plasmid conjugal DNA transfer proteins TraD, TraM, and TraI. Beth Traxler, Tran Nguyen, and Eliora Gachelet, Dept. of Microbiology, University of Washington, Seattle, Wash. 98195

A limited set of F plasmid proteins are involved in the DNA transfer stage of conjugation. These include the cytoplasmic proteins TraI and TraM and the cytoplasmic membrane protein TraD. While TraI has been well characterized as a *oriT* nickase/helicase, the functions of TraM and TraD are unknown. Furthermore, the nature of interactions between these proteins during DNA processing and transfer is largely uncharacterized. Previously, *in vitro* interactions between TraD and TraM and TraD and TraI have been reported. A more detailed analysis of these protein-protein contacts is critical to understanding the molecular events that occur during plasmid transmission from a donor to a recipient.

We have isolated a collection of mutations in the *traD* and *traI* genes using the transposons Tn*lacZ*/in and Tn*phoA*/in. This mutagenesis results in the creation of a set of in-frame 31-residue insertion mutants for protein structure/function analysis. We are using several of these mutants to probe the interactions between functional and nonfunctional TraD, TraM, and TraI derivatives. Furthermore, the characterization of the TraI mutants gives clues on the structure of this complex enzyme.

We have examined the *in vivo* interaction between TraD derivatives and TraM by co-immunoprecipitation techniques. Antibodies specific for TraM will specifically recover TraM in a complex with TraD from cell extracts solubilized with non-ionic detergent. Several conjugation defective TraD mutants are still proficient to interact with TraD, but at least one C-terminal TraD mutant is severely compromised in its ability to associate with TraM. Continuing studies will determine whether TraD and TraI also associate in an *in vivo* complex that can be recovered by this methodology.

# FEATURES OF THE pMV158-ENCODED PROTEIN MobM AND THE PLASMID ORIGIN OF TRANSFER, *oriT*

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The streptococcal plasmid pMV158 encodes the protein MobM, which is a relaxase involved in the initiation of its conjugal transfer among bacteria. Although pMV158 is not conjugative, it can be mobilised (albeit at a low frequency) by auxiliary plasmids that provide other functions needed for its transfer. Since pMV158 is a promiscuous plasmid, we have been able to transfer it among various hosts, Gram-positive and –negative. Transfer of pMV158 among streptococci and lactococci was mediated by plasmids belonging to the Inc18 family (pAMβ1), whereas transfer between *E. coli* strains was feasible by the use of IncQ (R388) and IncPα (RP4) plasmids. Uncoupling the *mobM* gene from the *oriT* region showed that MobM acts in trans. In addition, mobilization among *Escherichia coli* strains required at least two functions encoded by the auxiliary RP4 plasmid: the products of *traF* and *traG* genes. Protein MobM has been purified and shown to cleave a specific DNA sequence within the origin of transfer, the *oriT*, thus initiating the transfer. Analytical ultracentrifugation performed at various MobM concentrations showed that the protein is a dimer in solution. Dimerization could take place through a putative Leucine zipper that we have found in the amino acid sequence of the protein. In addition to the Leu-zipper, MobM has various conserved motifs, also found in proteins involved in DNA cleavage in conjugation (relaxases) and also in initiators of rolling circle replication (Rep initiators). Circular dichroism assays showed that MobM has a high content in alpha-helices (about 60%) and exhibits a melting temperature of about 40°C, which agrees with the protein having an optimal cleavage activity at 30°C, being reduced at 37°C. MobM-mediated cleavage of its DNA substrate is exerted at the sequence AGTGTG/TTA (being / the site of cleavage). The substrate of MobM is supercoiled plasmid DNA, leading to the appearance of plasmid-relaxed forms, although linear single-stranded oligonucleotides can be also cleaved by MobM. DNase I footprinting experiments showed that the region protected by MobM includes the *oriT* and the *mobM* promoter, suggesting that the protein auto-regulates its own synthesis. Fractionation of pneumococcal and of lactococcal cells harbouring pMV158 followed by immunological detection have allowed us to show that MobM is associated to the cell membrane. In order to monitor transfer of pMV158 between pneumococcal cells, we have constructed a gene cassette composed by the *gfp* gene (encoding the Green Fluorescent Protein) under the control of the pneumococcal promoter of the *malM* gene. Gene *malM* is regulated by the transcriptional repressor MalR (belonging also to the pneumococcal *mal* regulon), which is inactivated when the cells are grown in the presence of maltose as the carbon source. Consequently, expression of *gfp* is repressed in cultures grown in sucrose, whereas it is induced when the culture is shifted to maltose-containing medium. This cassette has been cloned into pMV158, and cells harbouring this plasmid were detected by fluorescence.

# INITIAL EVENTS IN SMALL STAPHYLOCOCCAL PLASMID TRANSFER

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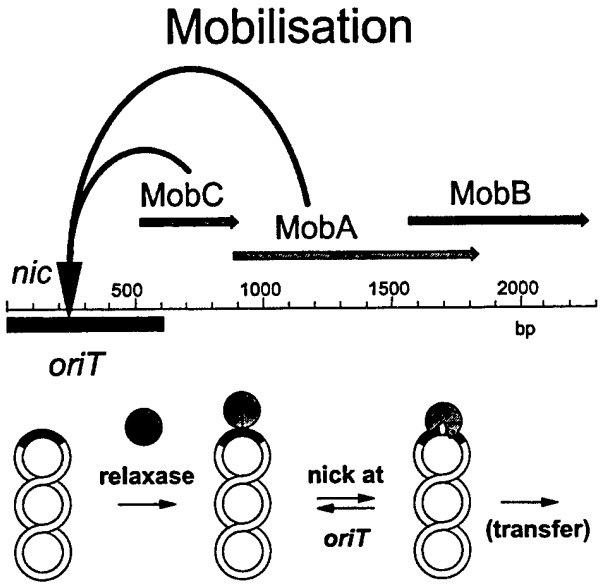
Horizontal gene transfer in bacteria results in genetic diversity with important medial consequences. Small non-self transmissible Staphylococcal plasmids such as pC221 and pC223 offer simple systems that embody the initial events in plasmid mobilisation. Plasmids pC221 and pC223 contain four loci involved in their mobilisation: an origin of transfer, *oriT*; a DNA relaxase, MobA; and the putative accessory proteins MobB and MobC.

Previous work has identified that the genes *mobA* and *mobC* are required for relaxase activity *in vivo* and are specific for their cognate *oriT* region. Preliminary activity studies on purified MobA showed that MobA alone was insufficient for nicking *in vitro*. To investigate the role of the MobC protein, the coding reading-frame from pC221*cop903* was isolated by PCR, cloned into pET vectors and expressed as both a native and $(His)_6$-tagged protein. Conditions required for optimum expression and purification have been optimised. Activity studies with purified MobC demonstrate a positive cooperative interaction with a 512 bp *oriT* fragment from pC221*cop903*. The MobC protein is currently undergoing crystallisation trials and molecular characterisation, details of which will be presented. Native and $(His)_6$-tagged MobA protein has been expressed and purification has been possible by both standard and novel approaches. *In vitro* nicking assays have demonstrated a requirement for MobA and MobC, in the presence of $Mg^{2+}$ or $Mn^{2+}$, for substrate- and site-specific nicking. Initial characterisation of the nicking reaction, using both cognate and non-cognate substrates, reveals some intriguing findings, details of which will be presented.



## DIMERISATION OF THE REPD INITIATOR PROTEIN IS A SPECIFIC AND STAGE-REGULATED PROCESS

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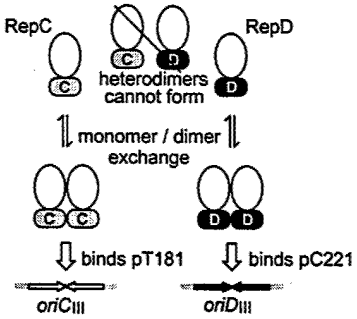
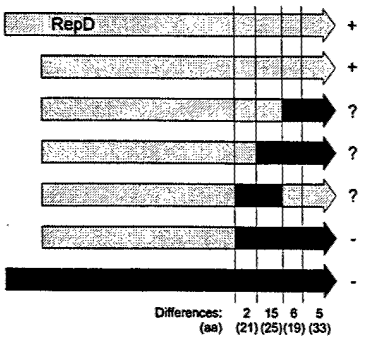
The dimeric RepD protein serves to initiate replication of staphylococcal plasmid pC221 by a rolling-circle mechanism. It is a close relative of the RepC protein specified by pT181: the two proteins are over 80% identical, yet the two plasmids are compatible, thanks to differences in sequence specificity by these initiators for their respective origins of replication. The proteins share common features for the nicking-closing reaction, and the sequence specificity can be switched by simple exchange of a divergent C-terminal domain of 14kDa, as has been found for the fusion protein RepDC.

Previously, studies were designed to investigate orientation of the RepD dimer and potential complementation of defective active sites by attempting to form heterodimers between RepD and RepDC. Different routes to heterodimer formation included combination of full-length (37kDa) and truncated (34kDa) forms, differential utilisation of purification tags, and even co-expression was attempted without success. But heterodimer formation *was* possible between 37kDa and 34kDa forms of RepD - and this has now been found to occur without the need even for co-expression or active site mutations - just mixing of the two forms.




This result indicates (a) that specificity for dimer formation resides within the C-terminal region of the Rep proteins, and (b) that the monomers must be in a rapid free exchange within the cell. An immediate implication concerns RepD and RepC *in vivo* when pC221 and pT181 are co-resident in the same host. By "forbidding" heterodimer (D:C) formation, hybrid proteins cannot form - which could otherwise influence the individual copy numbers of the plasmids. Can the area required for specific dimer formation be narrowed down? A series of crossover mutations between RepD and RepC has been constructed to address this point, and the latest results defining the dimerisation region will be presented.




If dimerisation is protein-specific prior to the stage of initiation, what about afterwards? Following replication, the Rep proteins are inactivated by formation of a heterodimer containing one "normal" subunit and one with a covalent DNA adduct (here designated RepD/D*). This adduct, an artifact of the termination process, is useful as a means to inactivate the protein (preventing further unwanted initiation). But surely if the monomers are in free exchange, then all the "good" (i.e. non-substituted) monomers will re-associate - which will lead to more replication until all the Rep protein is in the adduct form. Yet non-substituted and adduct forms are routinely detected in 1:1 ratio in extracts obtained from plasmid-containing cells.

So monomer exchange has been tested *in vitro* using artificially generated 34kDa RepD/D*. Results show that the covalent DNA adduct locks the protein subunits together. No evidence has been found for any heterodimer formation between a tagged 37kDa RepD subunit and *either* of the subunits comprising the RepD/D* heterodimer. This shows that there is no free 34kDa subunit available, even if one argues that the 34kDa adduct form is itself unable to re-associate with 37kDa RepD. The result makes sense for regulation of replication: there is little point inactivating the Rep protein if it will reactivate simply by re-assortment.

Thus the covalent adduct should not be seen simply as a passive end-product of the replication process - it is actually playing an active part in maintaining the RepD/D* heterodimer association. This leaves a final question of whether RepD/D* is targeted by the cell for degradation of both subunits together, ensuring turnover in equal proportions - a question which has yet to be addressed.

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A DNA INVERTASE SPOILS CONJUGATION FOR pVT745

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Plasmid pVT745 from *Actinobacillus actinomycetemcomitans* strain VT745 can be transferred to other *A. actinomycetemcomitans* strains at a frequency of $10^{-6}$. Screening of transconjugants revealed that the DNA of pDMG21A, a pVT745-derivative containing a Km-resistance gene, displayed a structural rearrangement after transfer. The isomeric form of the plasmid was designated pDMG21B. Results of restriction enzyme analysis revealed that a 9-kb segment on the plasmid had switched its orientation from A to B. The inversion was independent of RecA and required the activity of the pVT745-encoded site-specific recombinase. This recombinase, termed Inv, was highly homologous to invertases of the Din family. Two recombination sites of 22-bp, which are arranged in opposite orientation and which function as DNA crossover sequences were identified on pVT745. One of the sites was located adjacent to the 5' end of the invertase gene, *inv*. Inversion of the 9-kb segment on pVT745-derivatives has been observed in all *A. actinomycetemcomitans* strains tested except for the original host VT745. In fact, whereas VT745 harbors pDMG21A exclusively, only its isomeric form, pDMG21B, could be isolated from selected transconjugants. This would suggest that a host factor which is either inactive or absent in VT745 is required for efficient recombination. Inactivation of the invertase in the donor strain resulted in a 1000-fold increase in the number of transconjugants upon plasmid transfer. It was unlikely that $inv^+$ (pDMG21) and $inv^-$ (pDMG27) plasmids were transferred at different rates since electroporation experiments had indicated that Inv was neither active in the host VT745 nor activated by conjugation alone. When a spectinomycin resistance cassette was inserted into pDMG21A 1.8-kb outside the invertible segment (pDMG32) the number of transconjugants obtained increased by one order of magnitude when compared to transfer of pDMG21A. Results of restriction enzyme analysis with pDMG32 DNA isolated from selected transconjugants revealed the presence of the invertible region in both orientations, A and B. It appears that an additional plasmid-encoded element, which acts either in *cis* or in *trans* affects inversion of the 9-kb segment. Such element has to be located in the proximity of the *spc* insertion. It is proposed that the low number of transconjugants obtained after transfer of pDMG21A is the result of an immediate loss of the plasmid in most recipient cells caused by an activated invertase. At this point the presence of the *inv* gene on pVT745 would appear to be a disadvantage for the effective dissemination of the plasmid via conjugation. It remains to be seen if a true biological role can be associated with *inv*, or if the gene is the remnant of a functional element, which once was part of pVT745 but subsequently lost.

# *ENTEROCOCCUS FAECALIS* SEX PHEROMONE SYSTEMS: TRANSFER ORIGINS OF pAD1 AND pAM373 AND GENETIC ANALYSES OF THE CORRESPONDING PHEROMONES

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*Enterococcus faecalis* secretes multiple peptide sex pheromones that induce a mating response by donor bacteria carrying certain types of conjugative plasmids. pAD1 (60 Kb) and pAM373 (37 Kb) represent two families of plasmids, one of which encodes a response to the octapeptide cAD1 (LFSLVLAG) while the other responds to the heptapeptide cAM373 (AIFILAS). The plasmids are in different incompatibility groups and have been sequenced in their entirety (De Boever *et al.*, 2000; Francia *et al.*, 2001). While they bear similar genes related to a number of conjugation functions, there are differences in their regulation of the mating response. The presence of plasmid DNA in a given host generally shuts down or "masks" the endogenous sex pheromone, and a secreted plasmid-encoded inhibitor peptide acts to prevent self-induction *via* competition with pheromone -- probably on the donor surface. The inhibitor peptides iAD1 (LFVVTLVG; encoded by pAD1) and iAM373 (SIFTLVA; encoded by pAM373) block the responses to cAD1 and cAM373, respectively.

pAD1 has two transfer origins (An and Clewell, 1997; Francia *et al.*, 2001); *oriT1* is located within an essential plasmid replication gene (*repA*), while the other, *oriT2,* is located about 180° away on the circular element – between a gene corresponding to a "relaxase" and a "*traG*-like" determinant. One *oriT* has been identified on pAM373, in a position resembling that of *oriT2* of pAD1. Using pAD1, in-frame deletions of the two determinants flanking *oriT2* were found to eliminate transfer; and a specific *nic* site has been identified. The *oriT2* of pAD1 and *oriT* of pAM373 both contain a large inverted repeat (about 140 nt) adjacent to a series of short direct repeats, and in the case of pAD1 the relaxase (TraX) has been shown to nick within the inverted repeat. While the inverted repeat sequences of pAD1 and pAM373 are very similar, the direct repeats differ significantly. A cloned (pAM401 shuttle vector) hybrid *oriT* consisting of the inverted repeats of pAM373 and the direct repeat of pAD1 could be transferred *in trans* by pAD1 but not by pAM373, implying that specificity relates primarily to the direct repeats.

Recent completion of the *E. faecalis* genome by TIGR has revealed that the secreted pheromones represent processed forms corresponding to the last 7-8 amino acid residues of the signal sequences of lipoprotein precursors (Clewell *et al.*, 2000); and we have now further characterized *cad* (An and Clewell, 2002) and *camE* (Flannagan and Clewell, 2002), which encode cAD1 and cAM373, respectively. We have also identified a gene, *eep* (An *et al.*, 1999), which encodes a protein we believe to be a zinc metalloprotease involved in processing within the signal sequence of the *cad* product (at the amino terminus of what ultimately becomes cAD1) but not to that relating to *camE*. Mutants of *eep* do not produce detectable cAD1 but do produce cAM373. Interestingly, *eep* mutants were also defective in production of

iAD1 when pAD1 was present; however, there was little, if any, effect on the production of iAM373 from pAM373-containing cells. The corresponding lipoproteins did not resemble each other and did not have strong homology with any sequences in the database. Their functions do not appear to be essential for cellular growth under routine laboratory conditions, and mutants harboring plasmid DNA exhibited normal responses to exogenous pheromone.

A cAM373-like peptide is also produced by *Staphylococcus aureus* but differs at the C-terminal residue (an alanine rather than a serine). The related gene, *camS* (Flannagan and Clewell, 2002), encodes a lipoprotein that is completely different from the enterococcal *camE*; although, like cAM373, the *staph*-cAM373 peptide corresponds to the C-terminal portion of the related signal sequence. The lipoprotein precursor has homologues in *Staphylococcus epidermidis*, *Bacillus subtilis*, and *Listeria monocytogenes*, but the corresponding heptapeptides present within those sequences do not closely resemble *staph*-cAM373; and those species do not secrete a pheromone activity. pAM373 has been shown to transfer into, but not replicate in, *S. aureus,* although it can be maintained when cointegrated with the enterococcal resistance plasmid pAD2 (Showsh *et al.*, 2001) or the shuttle vector pAM401 containing a cloned *oriT*. The fact that a recently identified vancomycin-resistance (*vanA*) plasmid (pAM368) (Showsh *et al.*, 2001) confers a response to cAM373 (as well as *staph*-cAM373) suggests that vancomycin resistance, which has become prevalent in clinical isolates of enterococci, may eventually move from these organisms into *S. aureus*.

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Molecular and Genetic Dissection of the Mechanism of Negative Control of Pheromone-inducible Conjugation in the *Enterococcus faecalis* pCF10 System.

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*E. faecalis* cells carrying the conjugative antibiotic resistance plasmid pCF10 express transfer functions only when plasmid-free recipient cells are in close proximity. The plasmid-encoded sensing system detects a chromosomally-determined heptapeptide pheromone called cCF10, excreted into the growth medium by recipient cells. The cell wall fraction of recipients also contains a substantial quantity of cCF10, which may play a role in cell-cell signaling by direct contact, eg. when the organisms are growing in a surface environment, such as a biofilm. Recent results from several groups indicate that the peptide pheromones specific for several plasmids are all produced by proteolytic processing of signal peptide components of secreted lipoproteins. Since both donors and recipients have the genetic potential to produce cCF10, the plasmid also encodes a system to block self-induction of donor cells by endogenous pheromone. Negative control of conjugation functions in the absence of exogenous pheromone is mediated by four pCF10-encoded gene products. The inhibitor peptide iCF10, encoded by the *prgQ* locus, is secreted into the growth medium in an amount just sufficient to neutralize the cCF10 released by the donor cell. The membrane protein PrgY blocks self-induction of donor cells by endogenous cell-wall associated pheromone. Two additional cytoplasmic regulators, PrgX protein and Qa RNA also play critical roles in negative control.

The pheromone-induction process in the pCF10 system involves a complex sequence of events, many of which involve posttranscriptional processes. In recent years, we have focused on experimental strategies that allow for separation of initial events in pheromone induction from those occurring subsequently. Most if not all pheromone-inducible transcription of pCF10 originates from the promoter for *prgQ*. As noted above, this locus encodes the iCF10 peptide; it also encodes RNAs that play important regulatory roles. The *prgQ* promoter $P_Q$ is highly active under all conditions, although pheromone may cause a modest increase in its activity. In uninduced donor cells a predominant ~400 nt mRNA called QS is detected. The 3' end of QS corresponds with the position of an inverted repeat structure IRS1; this structure probably participates in termination or in processing of transcripts to generate QS. The most important difference in *prgQ* transcription associated with initiation of pheromone induction is the appearance of a relatively stable extended transcript QL, which contains about 130 extra nt at the 3' end. RNA sequences in the extended region of QL have been shown to play an essential role in the downstream steps of pheromone induction. Our recent results indicate that the combined activities of PrgX and Qa interfere with QL production in the absence of pheromone, and that PrgX comprises the pheromone-responsive molecular switch controlling the initial induction step.

Genetic analysis of the requirements for PrgX expression showed that both Qa and PrgX are produced from a transcript initiating in the QS coding region from a promoter called $P_{Qa}$, but on the opposite strand. Our analysis shows that the initial 120 nt of this transcript correspond to Qa, and that transcription can extend to a termination sequence at the 3' end of *prgX*. PrgX acts as a positive regulator of its own expression,

as well as that of Qa, probably by affecting the processing of transcripts from $P_{Qa}$. PrgX and Qa are both required for blocking production of *prgQ* transcripts extending past IRS1. PrgX binds to two sites in pCF10 DNA between the *prgQ* and *prgX* coding sequences, and DNA binding is required for both the positive autoregulatory activity of PrgX and for its activity as a negative regulator of conjugation. PrgX exists as a dimer in uninduced donor cells, and the addition of exogenous cCF10 disrupts the dimers *in vivo*; however cCF10 does not abolish DNA binding by PrgX. These data have been incorporated into a new molecular model for PrgX/Qa regulation of pCF10 conjugation. In order to test the model, a comprehensive analysis of these regulators using genetic, biochemical and structural analyses is being carried out. The new model and the results obtained to date will be presented.

PURSUING THE TARGET OF THE pAD1 *par* POST-SEGREGATIONAL KILLING SYSTEM. Keith E. Weaver, Carol Wells, Chris Waters, Dariel Weaver, Annie Rezic, and Tony Greenfield. Division of Basic Biomedical Sciences. School of Medicine. University of South Dakota. Vermillion, SD 57069

The pAD1 plasmid is the prototype of the pheromone-responsive conjugative plasmids native to *Enterococcus faecalis*. The pAD1-encoded *par* locus was the first non-restriction/modification associated post-segregational killing (PSK) system identified in Gram + bacteria. It is regulated by a unique antisense RNA mechanism that involves interaction between the toxin message and the antitoxin antisense at dispersed regions of complementarity at both the 5' and 3' ends. As in the *hok/sok* system the antisense RNA is less stable than the toxin message and inhibits toxin translation. As has been the case in many PSK systems, the target of the *par* toxin has proven elusive. Affinity chromatography and Far Western analyses have failed to reproducibly identify an interacting protein. Transmission electronmicrographs of cells exposed to elevated toxin levels showed multiple alterations in cell morphology including inappropriately positioned nucleoids, mis-segregated chromosomes, and aberrant cell division. Scanning electronmicrographs (SEM) revealed a specific defect in the timing of the initiation of cell division along with other division and cell wall abnormalities. Isotope incorporation studies showed that all macromolecular synthesis ceases nearly simultaneously 30 minutes after induction of toxin production, about 30 minutes before any effect on cell growth is observed. Interestingly, reducing growth rate by nutrient limitation reduces the effect of toxin over-production as measured by growth inhibition. Surprisingly, reducing growth rate by lowering culture temperature has the opposite effect, exacerbating the effects of the toxin.

Using a graded approach to toxin exposure, seven host mutants resistant to the effects of *par* toxin were isolated. Surprisingly, all seven mutants showed an increased sensitivity to glycine in the growth medium, which is often used to facilitate electroporation of *E. faecalis*. SEM revealed that, while glycine caused the swelling of wild-type cells with the production of many small pores, the same levels of glycine caused elongation and fracture of the mutants. SEM and flow cytometry showed that the mutant cells were slightly smaller than wild-type cells in the absence of glycine. Preliminary 2-D polyacrylamide gel analysis revealed multiple differences in the proteome of the mutant and wild-type strains. Growth curve and toxin inhibition analysis revealed that six of the seven mutants showed identical characteristics, with a slightly reduced growth rate in the absence of toxin and nearly complete toxin resistance. Despite repeated efforts, toxin resistance could not be increased in these mutants. The seventh mutant, which had been exposed to the least toxin, showed no difference from wild-type in growth in the absence of toxin, but was more sensitive to toxin and less sensitive to glycine than the other mutants. Therefore, we believe that resistance to toxin occurs in at least two steps, with the seventh mutant representing an intermediate to the other six.

# CONJUGATIVE TRANSFER OF BROAD-HOST-RANGE PLASMIDS IN GRAM-POSITIVE BACTERIA

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Conjugation in Gram-negative bacteria has been studied in great detail in the last decades, whereas the transfer mechanisms of antibiotic resistance plasmids in Gram-positive bacteria remained obscure.

Recently, the entire nucleotide sequence of three large broad-host-range conjugative plasmids, the staphylococcal pSK41 (1), the lactococcal plasmid pMRC01 (2) and the *Enterococcus faecalis* plasmid pRE25 (7) as well as the transfer regions of two further conjugative plasmids, pGO1 (6) and pIP501 (4, 5) have been determined. The analyses of their sequences revealed similarities of their respective transfer genes extending the first six genes as described previously (1, 2) but also several interesting similarities to type IV secretion systems. Type IV secretion is involved in bacterial conjugation in Gram-negative bacteria as well as in protein export of effector molecules to eukaryotic target cells. These similarities with type IV systems and homologies of the DNA relaxases and their targets (*oriT*s) with well studied Gram-negative conjugation systems will be discussed in detail. They permit first speculations on a possible mechanism for plasmid transfer in unicellular Gram-positive bacteria (3).

Our model plasmid is the 30.2-kb broad-host-range conjugative multiple antibiotic resistance plasmid pIP501 belonging to the incompatibility group Inc18. Eleven pIP501-encoded transfer genes were proven to be organized in an operon with the first gene representing the DNA relaxase TraA (4, Fig. 1). TraA was overexpressed in *E. coli* and purified via affinity chromatography. GST-TraA-mediated site- and strand-specific cleavage of supercoiled pIP501 *oriT* DNA was shown to be strictly dependent on divalent cofactors and was highest at temperatures of between 42 and 45°C (4).

Data on the organization of the remaining putative pIP501 transfer factors and the regulation of the main transfer operon *orf1-11* will be presented.

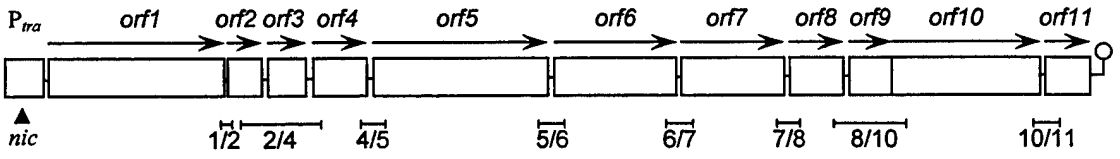


**Fig. 1.** Organization of the *tra* region of pIP501 encompassing *orf1-11*. Open reading frames are shown as boxes with horizontal arrows indicating the direction of transcription. The *oriT* nick site is indicated by a wedge. RT-PCR products that proved cotranscription of *orf1-11* are shown below the diagram. They are named according to the genes amplified, counting from *orf1-11*.

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GENETIC ANALYSIS OF THE ORIGIN OF TRANSFER ON PLASMID pVT745

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pVT745 (25kb) is a conjugative plasmid that was isolated from the Gram-negative pathogen, *Actinobacillus actinomycetemcomitans*. It has been shown that pVT745 transfers to other *A. actinomycetemcomitans* strains and mobilizes a co-resident non-transmissible plasmid, pMMB67, at a frequency of $10^{-6}$. The origin of transfer (*oriT*) is a *cis*-acting site located on conjugative or mobilizable plasmids, which is required to initiate transfer-associated DNA replication by the introduction of a site- and strand-specific nick. Origins of transfer are typically positioned in non-transcribed intergenic regions, show a higher AT content than their surrounding DNA and have extensive direct and inverted nucleotide sequence. Various families of *oriT* sequences which show strong nucleotide conservation in the vicinity of the nicking site have been identified. However, no homology was detected between known *oriT* sites and pVT745. Nonetheless, we assumed that the pVT745-specific *oriT* was located in a 1-kb region just upstream of the operon *magA* which encodes genes required for transfer-associated replication such as the nickase or relaxase. This locus shared some but not all of the characteristics typically associated with *oriT* sites, including the presence of inverted repeats. To identify the pVT745-specific *oriT* several fragments containing the region 5' of the *magA* gene cluster were cloned and tested for mobilization by a kanamycin resistant derivative of pVT745 (pDMG27) as the auxiliary plasmid. Two clones carrying a 2.8-kb (pJC1) and 1.1-kb insert (pJC2) appeared to be mobilized to *Escherichia coli* and *A. actinomycetemcomitans* strains in surface mating experiments based on marker selection. Smaller subclones were not transferred. However, mobilization of pJC1 and pJC2 seemed to be associated with the simultaneous transfer of pDMG27 despite the use of RecA-deficient donor cells and the inactivation of a pVT745-encoded recombinase. Since neither pJC1 nor pJC2 could be mobilized independently it was arguable that either clone carried *oriT*. An attempt to use an RK2-derivative as auxiliary conjugative plasmid for mobilization of pJC1 and pJC2 failed. Therefore, a different strategy was used to identify the origin of transfer. If the mating event itself was to be interrupted prematurely before an intact copy of pVT745 was transferred to the recipient cell such truncated plasmid copies would have to carry identical segments representing the 5' end of the transferred linearized form of the plasmid. These ends would denote the site where nicking had occurred and therefore be part of *oriT*. To monitor such interrupted transfer two markers were placed on pVT745, resulting in construct pDMG27. A streptomycin resistance cassette was inserted 5' of the putative *oriT*, and a kanamycin resistance cassette 3' of the putative *oriT*. After reducing mating times from the original 6 hours to 5 minutes two transconjugants were obtained which were Km resistant and Sm sensitive. They both harbored a truncated form of pDMG27. Results of restriction enzyme analysis and DNA sequencing confirmed that the nicking site was located just upstream of the *magA* operon and had been present on pJC1 and pJC2 all along. It reads TTCTACATT with the nick occurring between A and C. An identical inverted repeat of this sequence was located 56-bp downstream of the nick site. The location of *oriT* will be confirmed by the insertion of

mutations into the nick region sequence and subsequent monitoring of transfer frequencies of such mutated plasmids.

TRANSCRIPTIONAL REGULATION OF R100 TRANSFER GENE EXPRESSION

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The existence of several different regulatory systems that act upon the transcription of the very similar transfer operons of F, R1 and R100 is well established now. These include things like the DNA binding proteins: TraJ, ArcA, TraM, TraY and RfaH, and their respective binding sites: *sbj, arcA, sbm, sby* and *ops*. Additionally, there is the FinO-*finP* antisense pair that blocks translation of traJ transcripts; strong secondary structures in the traL transcript that stabilize traA transcripts; and one or more internal promoters. Presently the consensus is that transcription begins at one or more sites near *traY* and has the potential of continuing all of the way through *traX*, just 5' of *finO*. How much is made and how long the transcripts are depends upon the actions of the different regulatory elements.

Not all of the above sites have been specifically shown to function in R100, but because of the close homologies and frequent identities in sequences among F, R1 and R100, sites that have been specifically shown to function in one of the plasmids are reasonably presumed to function in the others. In R100, the *arcA* binding site and the *ops* site are both known from sequence analysis but have not been specifically shown to function in transcription of the R100 transfer genes.

The present work was undertaken to explore the *ops* site in R100-1 (a *finO* mutant of R100) and also to determine if the sequence, aaGCTCTTa (lysleuleu), that can be found inside the R100-1 traY ORF was important for the normal expression of the R100-1 transfer genes. The capitalized bases encode an RNA-protein binding site for the well-known BoxA protein found in bacteriophage lambda transcriptional regulation. The above site was mutated to aaatTacTc (lysleuleu) and the *ops* site was separately mutated and also deleted. The mutant R100-1 plasmids were analyzed for their abilities to transfer. The transcripts produced by each were analyzed by northern blots that were probed with specific RNA probes for *traY, traK, traV, traR,* and *traG*. Probing for other *tra* genes is in progress now.

The putative BoxA binding site with alternate codons proved to have no detectable effect on the transferability of R100-1. There was also no detectable effect on the amount of *traY* transcripts that could be seen in northern blots when the mutant was compared to the parental R100-1. It is possible that some small differences exist in the amounts of *tra* transcripts detectable by probes for distal *tra* genes such as *traG*.

In R100 the putative *ops* site is found inside the transfer operon rather than 5' to it as in other operons where *ops* functions. In R100, *ops* is in the intergenic space between *traV* and *traR*. Replacing the *ops* site with a mutant *ops* site, or deleting it entirely, proved to have a major effect on transfer. Both types of mutants had extremely low levels of transfer similar to those we reported for R100-1 deletion mutants lacking the *traJ* gene. As anticipated, transcription of distal *tra* genes in these mutants was not detectable with the *traG* probe.

One conclusion from these studies is that the *ops* gene of R100 is required for transfer and for transcribing the distal *tra* genes. The other is that the putative BoxA binding site in *traY* is not a true BoxA site.

# ASSEMBLY REQUIREMENTS OF TRHC-ASSOCIATED PROTEIN COMPLEXES WHICH ARE INVLOVED IN THE CONJUGATIVE TRANSFER OF R27

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We have previously determined the cellular location and temperature-dependent assembly kinetics of protein complexes comprised minimally of TrhC, an inner-membrane associated R27 transfer protein homologous to Ti plasmid VirB4, F plasmid TraC, and IncP TrbE (1). Using a green fluorescent protein (GFP) fusion construct, TrhC-GFP was detected during fluorescence microscopy of live bacteria at the periphery of cells as discrete foci. Our hypothesis is that these foci represent protein complexes that participate in the conjugal transfer of plasmid R27. We are now extending the above study to determine the assembly requirements and composition of these protein complexes. Firstly, we have developed an assay to determine which R27 transfer proteins are necessary for TrhC-GFP to enter into protein complexes. By expressing TrhC-GFP in cells containing R27, foci are detected; however in cells lacking R27, cells are a confluent green. Using R27 transfer mutants (in which a single transfer gene has been disrupted) we can determine which transfer proteins participate in complex assembly. So far we have found that TrhL, TrhE, TrhK, TrhB, and TrhV are each required for fluorescent foci formation. We hypothesize that these transfer proteins interact either directly or indirectly with TrhC in a multi-protein complex where individual protein-protein interactions are essential for either the assembly and/or the stability of the complex. In addition, we have created point mutations in both *trhC* and *trhC-gfp* through site-directed mutagenesis to determine which residues/motifs contribute to complex assembly and DNA transfer. In particular, the Walker A and B motifs involved in nucleotide triphosphate binding are present in TrhC (Figure 1), and we have concentrated our mutagenesis within these regions. The goal of this study is to characterize the R27-encoded conjugative apparatus by determining protein-protein interactions and the specific amino acids required to maintain those interactions and plasmid transfer.

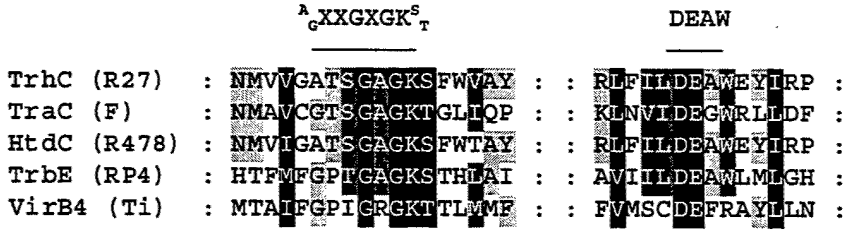


**Figure 1.** CLUSTAL alignment of two conserved regions within VirB4 family proteins proposed to play a role in nucleotide binding/metabolism. Plasmid sources are in brackets.

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**Characterization of the coupling protein of the IncHI1 plasmid R27.**

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The TraG family of coupling proteins is thought to enable conjugative transfer of plasmid DNA by facilitating an interaction between the relaxosome and mating pair formation complex within the donor bacterial cell. TraG is the putative coupling protein of R27, the prototypical plasmid of the IncHI1 family. Using insertional mutagenesis we have shown that TraG is essential for conjugative transfer, and yet not involved in the propagation of the H pilus-specific phage, Hgal, suggesting this protein plays no role in pilus assembly, but rather DNA translocation (1). Protein sequence alignments with the TraG-like family revealed two conserved regions which have been proposed to be the nucleoside-triphosphate binding motifs Walker A and B (Figure 1). Site-directed mutagenesis within the Walker A box (G210D) and Walker B box (E511K) revealed a 100 and 1300 fold decrease in conjugative ability, respectively. Continuing mutagenesis studies are underway to identify amino acids within these conserved regions that are involved in conjugative transfer. In order to localize the R27 coupling protein, we have fused both $His_6$ and GFP to the C terminus of TraG on the R27 plasmid. This offers us the substantial advantage of visualizing TraG at native levels, since the fusions are under control of the natural expression determinants. The TraG fusions are both proficient in conjugative transfer, reaching wild type levels. Using fluorescent microscopy, the coupling protein was found to distribute evenly to all regions of the cell, as uniformly green cells were observed. Membrane association of TraG-$His_6$ was revealed by immunoblot analysis of membrane fractions. Based on these observations we conclude that the coupling protein from R27 is located in the membrane throughout the donor cell raising the possibility that conjugative transfer of plasmid DNA can occur from any position of the donor cell.

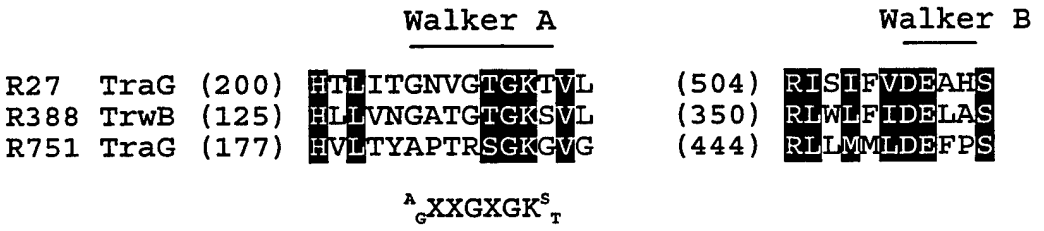


Figure 1. Alignment of Walker A and Walker B boxes from coupling proteins from R27 (TraG; accession NP_058332), R388 (TrwB; accession CAA44852), and R751 (TraG; accession S22992). Motifs were aligned in MegAlign (DNASTAR Inc.) and shaded in GeneDoc using conservative mode and 2 levels (1).

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MOLECULAR ANALYSIS OF THE SEQUENCE-SPECIFICITY DIFFERENCES BETWEEN R100 AND F FACTOR TRAI NUCLEASE DOMAINS

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Relaxases are necessary for the initiation and termination of conjugative DNA transfer. These plasmid-encoded proteins create a site-specific nick in the plasmid DNA by forming a covalent attachment to one of the plasmid strands via a phosphotyrosyl linkage. Upon termination of conjugation, the relaxase performs the reverse reaction to ligate the cleaved plasmid strand. In F factor TraI, R100 TraI, and other related relaxases, the relaxase function is linked to a helicase function. Physical linkage of the two proteins is required for efficient transfer. The relaxase activity of TraI is contained within the N-terminal 330 amino acids in a domain referred to as TraI36. F factor TraI36 recognizes an eleven-base single-stranded DNA sequence with high sequence-specificity. F factor and R100 TraI36 share 91% amino acid sequence identity, yet these two proteins recognize different DNA sequences (5′-GTGGGGTGTGG-3′ for F factor and 5′-GTAGTGTGTGG-3′ for R100). To identify the amino acid residues responsible for the specificity difference, each non-identical residue from R100 was placed in the F factor background by the generation of chimeric proteins containing six or nine substitutions, or mutant proteins with one to three substitutions. Binding specificity was determined by following anisotropy and intensity changes of specific, fluorophore-labeled oligonucleotide upon titration of mutant protein. The great majority of mutants show no significant specificity change. The single mutants Gln192Arg and Arg201Gln have lost specificity for F factor origin of transfer (*oriT*) oligonucleotide, with binding affinities reduced by 3 orders of magnitude, yet these mutants have not gained R100 binding specificity (no detectable increase in binding affinity for R100 *oriT* oligonucleotide). Subsequent experiments will test whether the Gln192Arg/Arg201Gln double mutant generated by swapping Arg and Gln in the primary sequence is sufficient for the two-base specificity difference. Also, we are currently performing *in vitro* nicking assays with the mutant proteins to further investigate the difference in specificity.

# PEPTIDOGLYCAN HYDROLYSIS BY P19 AND RELATED PROTEINS FROM TYPE III AND TYPE IV SECRETION SYSTEMS.

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In bacteria the cell wall (or peptidoglycan, or murein sacculus) does not only function as a stress-bearing and shape-forming determinant of the bacterial cell but also imposes a physical barrier upon transport of macromolecules through the cell envelope (5). In *Escherichia coli* Slt70 (the soluble lytic transglycosylase) is one of the best characterised muramidases in *E. coli* which are involved in maintaining the integrity and plasticity of the cell wall of this organism. The protein has been crystallised (12) and a conserved fingerprint typical for lytic transglycosylases has been proposed (6). Sequence similarity searches revealed that this sequence fingerprint is present in an even larger family of proteins (3, 5, 7-9). Intriguingly, the newly identified members of the lytic transglycosylase family (based on sequence similarity) are either encoded by conjugative DNA transfer systems, virulence factor export systems (type III and type IV secretion systems), or bacteriophages. Thererfore, it seems that despite the large number and possible redundancy of these enzymes, macromolecular transport systems encode their own specialised lytic transglycosylases (the current homologous sequences are available in the PFAM database (1), http://www.sanger.ac.uk/Software/Pfam/, searching the text for "SLT", the PFAM accession number is PF01464).

What might be the role of these *specialised* lytic transglycosylases in bacterial trans-envelope transport? To address this question our group has been primarily interested in characterising the function of one member of this protein family, P19, in the conjugative DNA transfer of the F-like resistance plasmid R1. Earlier experiments from our laboratory revealed that protein P19 is necessary for efficient conjugal DNA transfer and R17 phage infection (3). Since it was found that P19 is neither involved in transfer gene expression nor assembly of the R1 sex-pilus, it was suggested that the protein plays a role in the DNA/RNA transport process itself (3). In our working model P19, which was shown to be transported into the periplasm (2), is associated with the proposed envelope-spanning transport complex. Its suggested function is a spatially and temporally defined opening of the peptidoglycan meshwork to allow the efficient formation of an active DNA transport complex. Indeed we could demonstrate that P19 can locally disrupt the cell envelope of *E. coli* upon over-expression (4).

P19 homologs in type IV secretion systems are VirB1 (Ti plasmids of *Agrobacterium tumefaciens*), TraL (IncN plasmid pKM101, PilT (IncI plasmid R64), TrbN (IncP plasmid RP4), VirB1 (*Brucella suis virB* operon). Disruptions of the respective gene led to a distinct decrease in the function of the system that was investigated, for example reduced tumor formation in the case of the Ti-plasmid *virB1* or reduced (10-100 fold) DNA transfer frequencies in the case of most of the P19 homologs encoded by conjugative plasmids were found. An explanation for the non-essentiality of these genes might be the intrinsic flexibility and plasticity of the peptidoglycan which certainly allows the formation of active transporters even in the absence of specialised lytic

transglycosylases, albeit with a much lower efficiency. Interestingly, the HP0523 protein encoded by *Helicobacter pylori* which only shows a weak homology to the specialised lytic transglycosylase family was found to be essential for CagA secretion (10).
Although our observations and experimental evidence from other laboratories strongly substantiate the idea that specialised lytic transglycolylases like P19 are important for bacterial trans-envelope transport, more data are required to characterise the role of these enzymes. Until now evidence for the proposed glycolytic activity of these proteins obtained by zymogram analyses is only available for the PRD1 phage VP7 protein (11). In our laboratory we are presently trying to confirm and extend our previously obtained results by using suitable expression/purification systems in combination with zymogram analyses and a newly developed ELISA to directly show the muramidase activity of selected proteins from the specialised lytic transglycosylase family. Several candidate proteins that were over-expressed in *E. coli* and purified were found to exhibit the expected peptidoglycan lysing activity, among them P19/ORF169 (Plasmids R1 and F), VirB1 (*B. suis* chromosome), VirB1 (*A. tumefaciens* Ti plasmid), HP0523 (*H. pylori* chromosome), IagB (*S. enterica* chromosome) and IpgF (*S. flexneri* plasmid pWR100). Interestingly, some of the proteins which were subjected to purification were found to interact with the chaperonin GroEL. Whether this interaction is specific and required for the assembly of the transport apparatus will be investigated.

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# THE *NIC*-SITE OF PLASMID R388 *ORIT* CONTAINS DIFFERENT DETERMINANTS FOR TRWC STABLE BINDING AND *NIC*-CLEAVAGE

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TrwC is a bifunctional enzyme that displays two biochemical activities essential for plasmid R388 conjugation: *oriT*-specific DNA strand-transferase (3) and DNA helicase (2). The N-terminal domain of the protein contains the relaxase activity, while the C-terminal domain is responsible for the DNA-helicase activity (4). In conjugative DNA processing promoted by TrwC two specific tyrosil residues, Tyr18 and Tyr26 are involved. Tyr18 catalyzes the first strand cleavage, while Tyr26 is involved in the strand transfer reaction that terminates the reaction (1). TrwC recognizes *nic*, a specific site within plasmid R388 *oriT*, and catalyzes a series of concerted *nic*-cleavage and strand transfer reactions. These reactions can be mimicked in vitro by the use of *nic*-containing oligonucleotides. CEMSA analysis demonstrated that the N-terminal 293 amino acid residues of TrwC, TrwC-N293, discriminate between the R388 *nic*-specific oligonucleotide and similar size sequences corresponding to *nic* sites of related plasmid *oriTs*. The stoichiometry of complex formation was examined by analytical ultracentrifugation. It was shown that a monomer of TrwC-N293 binds a single oligonucleotide molecule with a $K_D$ value of 0.6 μM. In addition, CEMSA results showed that for an oligonucleotide to bind tightly, a complete $IR_2$ sequence was required. Sequences immediately adjacent to the *nic*-cleavage site, both 5' and 3', were not required for *nic* binding. TrwC-N293:oligonucleotide complexes were remarkably stable. They could be isolated by gel filtration chromatography, and displayed a half life of over 10 hours. Experiments carried out with the same oligonucleotides previously used for binding analysis indicate that *nic*-cleavage requires only sequences contained between $IR_2$ and the *nic* site. TrwC binds this region in an unstable and transient manner that is enough for recognition and *nic* cleavage. Thus, it can be assumed that DNA binding and *nic*-cleavage by TrwC are independent processes that require different recognition sequences. We propose that TrwC binds first to *nic* by specific interaction with $IR_2$. Subsequently a different region of the protein is involved in proximal *nic* recognition and cleavage.

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F FACTOR TraM OLIGOMERIZATION

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F factor TraM, a DNA-binding protein, is essential for efficient bacterial conjugation. TraM is proposed to play a role in transmitting the mating signal to initiate conjugation; however, the identity of the signal, the mechanism of its action, and the role of TraM in this process are unclear. We present the first reversible denaturation of F plasmid TraM, a prerequisite for application of thermodynamic models that describe stability and oligomerization. The observed unfolding process, followed either by circular dichroism or intrinsic tyrosine fluorescence, is biphasic. Analysis by analytical ultracentrifugation strongly suggests that during the first transition the tetramer dissociates into monomers, which then unfold to the denatured state. Using limited proteolysis, we have identified a stable C-terminal domain of TraM. Neither this domain nor the remaining N-terminal peptide individually can complement a *traM* deletion mutant or bind DNA *in vitro*. Both TraM fragments, however, are able to oligomerize as shown in analytical ultracentrifugation experiments. These experiments suggest that functional TraM tetramers are formed from pre-folded monomers, and that both N- and C- terminal structures are involved in oligomerization.

# A BIOCHEMICAL AND GENETIC ANALYSIS OF THE TRANSESTERASE ACTIVE SITE IN THE F PLASMID TRAI PROTEIN

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The product of the *Escherichia coli* F plasmid *traI* gene (DNA helicase I) is required for DNA transfer via bacterial conjugation. This bi-functional protein catalyzes the unwinding of duplex DNA and is a sequence-specific DNA transesterase. To address the role of the TraI helicase activity in conjugative DNA transfer, *traI* mutants were constructed and their function in DNA transfer was evaluated using genetic and biochemical methods. Based on these studies, we conclude that TraI-catalyzed helicase activity is essential for DNA transfer.

The site- and strand-specific nick that is essential for initiation of DNA transfer occurs via a transesterification catalyzed by TraI protein. This results in the covalent linkage of TraI to the 5'-end of the transferred DNA strand. Studies of transesterases, such as RP4 TraI, have shown that a tyrosine is most likely the active site residue. Peptide sequencing of F-encoded TraI covalently attached to the DNA revealed two tyrosines as potential active site residues. Point mutations have been made to analyze the role of each tyrosine in conjugative DNA transfer. Current studies focus on the biochemical activities of the mutant proteins and their ability to complement a *traI* deletion in conjugation. Preliminary experiments indicate both tyrosines play a role in conjugative DNA transfer.

CHARACTERIZATION OF THE TRA1 AND TRA2 CONJUGATIVE TRANSFER REGIONS OF THE INCHI1 PLASMID R27: DEFINING A TYPE IV SECRETION SYSTEM SUB-FAMILY.

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The IncHI1 plasmid R27 contains two distinct regions essential to conjugative transfer: Tra1 and Tra2 (1). Each transfer region was investigated by DNA sequence analysis, mutagenesis of individual open reading frames (ORFs), genetic complementation of transfer-deficient mutants and an H-pilus specific phage assay. Of the 14 ORFs in Tra1, 9 were found to be essential for conjugative transfer (2). Tra1 (17kbp) contains genes encoding mating pair formation (Mpf) proteins, the coupling protein (TraG), components of the relaxosome, and the origin of transfer (*oriT*). Of the 24 ORFs in Tra2, 11 were found to be essential for transfer. The Tra2 region (33 kbp) included the majority of the Mpf genes and two separate, active partitioning modules. Thirteen of the IncHI1 Mpf proteins have homologs in the IncF conjugative transfer system, while the peptidase and relaxase have homologs in the IncP system. Thus, IncH1 plasmids appear to possess a chimeric transfer system in which components of other conjugative systems have been obtained over their evolution, highlighting the modular nature of conjugative transfer systems. The Mpf systems of IncHI1 and IncF plasmids encode for several transfer proteins that are absent in the VirB/IncP systems and lack a VirB11/TrbB homolog, suggesting that IncHI1/IncF Mpf systems constitute a type IV secretion system sub-family.

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THE MOBILIZATION OF THE SYMBIOTIC PLASMID OF *Rhizobium etli* CFN42 DEPENDS ON THE TRANSFER OF pCFN42a WHICH IS REGULATED BY QUORUM SENSING.

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*R. etli* CFN42 is able to produce nitrogen fixing nodules on roots of *Phaseolus vulgaris* plants (bean). This strain contains six plasmids designated pCFN42a to pCFN42f. pCFN42d has been identified as the symbiotic plasmid (pSym), because it carries most of the genes required for nodulation and nitrogen fixation.

We have evaluated the transfer frequency of each of these plasmids and we have found that the pCFN42a is the only one capable of performing self-transfer at high frequency ($10^{-2}$). This plasmid also functions as a helper for the mobilization of the pSym which is able to mobilize only in presence of pCFN42a.

Hybridization experiments against a cosmid library of pCFN42a, using the *tra* genes from pTiC58 of *Agrobacterium tumefaciens* as probe, allowed us to isolate a cosmid that is able to perform self-transfer, consequently, it contains all required elements for pCFN42a mobilization. We have named this cosmid as C-13.

We have sequenced C-13 and we have found homologous genes to all the *tra* and *trb* genes reported for pTiC58 but we have not found any genes related with opine catabolism. We also found, upstream of *tra*R, an ORF homologous to *cin*R, a LuxR-like regulator identified in pRL1JI of *Rhizobium leguminosarum*. In order to analyze the regulatory mechanism of the pCFN42a transfer, we constructed mutants of all regulatory genes as well as transductional fusions with a glucuronidase reporter. All the fusions (*tra*R, *tra*I, *tra*M and *cin*R) are expressed in a wild type background.

On the other hand, a derivative strain with a mutation in the *tra*I gene abolished the transfer ability of pCFN42a and the production of an N-acyl-HSL of 8 carbons which is produced in the wild type. Other N-acyl-homoserin-lactones are produced in this strain but they seem not to be involved in the transfer process. A mutant in *tra*M apparently has not effect on transfer frequency, but in a strain containing the *tra*M gene of pNGR234a of R. sp. NGR234 the transfer frequency of pCFN42a decreases, therefore, TraM is not a negative regulator in this system or at least it is not so strong. We are currently characterizing mutants in *cin*R and *tra*R genes.

Since an Acil-HSL seems to be involved in pCFN42a mobilization, and pSym depends on this plasmid for its transfer, we questioned whether transfer of both plasmids is regulated by the population size. We found that the transfer frequency of pCFN42a depends directly on the culture density and that pCFN42d (pSym) is able to mobilize only when pCFN42a has obtained its highest frequency. Therefore Qurom-sensing seems to regulate directly the pCFN42a transfer and indirectly the transfer of symbiotic plasmid.

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# DNA transfer and gene conversion in *Mycobacterium smegmatis*

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Conjugal transfer of chromosomal DNA between different isolates of *Mycobacterium smegmatis* has been demonstrated previously (1). To identify genes and *cis*-acting sequences essential for transfer, a cosmid library of chromosomal DNA from a transfer-proficient donor, $mc^2155$, was constructed. Cosmid clones that had acquired transferability were selected by a conjugation assay. One of these cosmid clones, cTra12, could transfer from $mc^2155$ to a recipient at a frequency of ~$1x10^{-5}$, which is similar to that of chromosomal transfer. Although we detected homology to cTra12 sequence in the recipient ($mc^2874$) chromosome by Southern hybridization, transfer from $mc^2874$ to $mc^2155$, or other recipients, was never detected. The DNA sequence of cTra12 was determined and showed to contain 32 potential ORFs. None of these ORFs has similarity to any known transfer genes, or is predicted to encode a gene that might be associated with transfer. Further analysis of cTra12 suggests that DNA transfer in *M. smegmatis* is atypical. We have failed to isolate a small *cis*-acting locus (equivalent to *oriT*) required for transfer and instead have defined several regions up to 5kb in size that mediate transfer. We have termed these *bom* sites (basis of mobility) to distinguish them from classical *oriT* sites. Unexpectedly, we have also found that the recipient must be $rec^+$, suggesting that *bom* sites may have lost the ability to recircularize following transfer. Finally, we have shown that DNA transfer is associated with gene conversion in the recipient, and is likely required for plasmid recircularization.

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## CHARACTERIZATION OF GENES INVOLVED IN THE MODULATION OF CONJUGAL TRANSFER OF THE *BACTEROIDES* CONJUGATIVE TRANSPOSON, CTnDOT

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In previous studies we identified an 18 kb region from the *Bacteroides* conjugative transposon CTnDOT that was sufficient for mobilization of co-resident plasmids and unlinked integrated elements as well as self-transfer from *Bacteroides* to *E. coli*. When this 18 kb region was cloned on a plasmid (pLYL72), the plasmid transferred itself constitutively in the absence of a co-resident conjugative transposon. However, when this plasmid was present in a *Bacteroides* strain containing a co-resident conjugative transposon, conjugal transfer was repressed in the absence of tetracycline, and enhanced in the presence of tetracycline. Results suggested that a negative and a positive regulator of conjugal transfer were encoded outside the transfer region of the CTnDOT element. In this work, a minimal and inducible transfer system was constructed and used in transfer and western blot analyses to identify the differentially regulated genes from CTnDOT responsible for the enhancement and repression of pLYL72 conjugal transfer. Both of these regulatory functions have been localized to a region of the CTnDOT element that is essential for CTn excision. In the presence of tetracycline, regulatory protein RteC activates the expression of a putative topoisomerase gene, *exc*, which in turn results in an increase in transfer protein expression and a concomitant 100- to 1000-fold increase in the frequency of pLYL72 transfer. Our results also suggest that since *exc* alone cannot result in enhancement of transfer, other factors encoded upstream of *exc* are also required. Conversely, in the absence of tetracycline, a gene located near the 3'-end of *exc* is responsible for the repression of transfer protein expression and also results in a 100- to 1000-fold decrease in the frequency of pLYL72 transfer.

HOST-MEDIATED CONTROL OF F PLASMID TRANSFER BY FIS AND IHF

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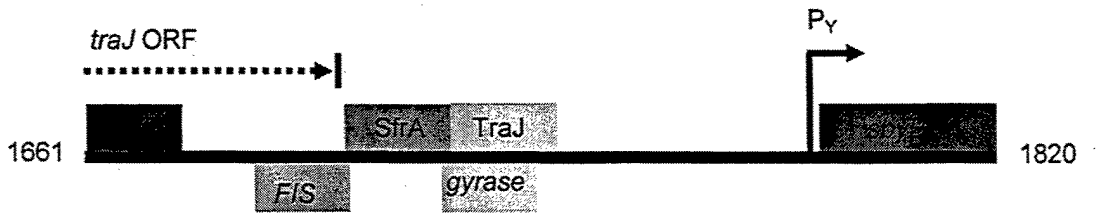
Bacterial mating allows the transfer of DNA from a donor cell to a recipient cell. The transfer is usually facilitated by the formation of a mating bridge between two cells, requiring close physical contact. The mechanism of transfer for the conjugative F plasmid in *Escherichia coli* has been the subject of much research. The initial activator of transfer gene expression in the F plasmid is thought to be the plasmid-encoded protein, TraJ, which activates the expression of the 33 kb transfer (*tra*) operon, containing most of the genes necessary for plasmid transfer. The *tra* operon includes *traY*, another regulatory protein which up-regulates expression of the entire operon (Frost *et al.*, 1994).

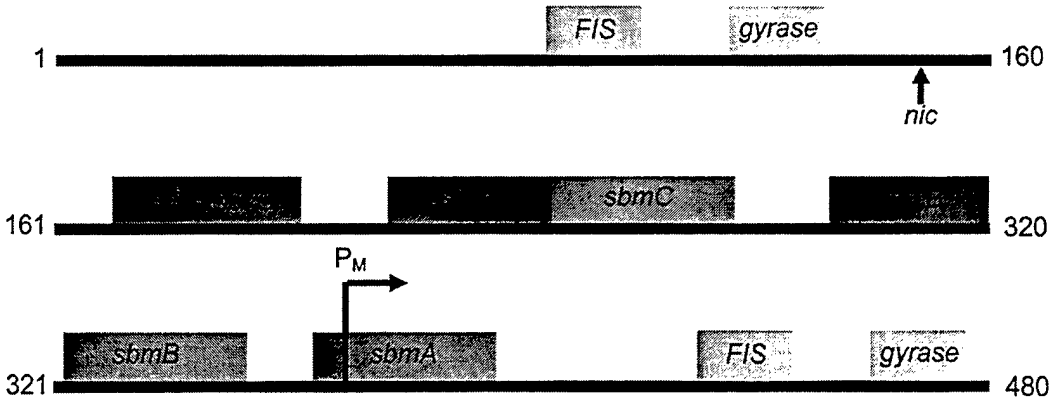
The mating ability of the F plasmid has been shown to peak early in exponential phase (Frost and Manchak, 1998). Mating then decreases, and eventually ceases as cells enter stationary phase. However, studies have shown that TraJ is still present long after donor cells have entered stationary phase. Conversely, TraY levels appear to decrease as cells enter stationary phase, suggesting the presence of an unknown repressor of *tra* operon expression. Previous studies have suggested the presence of a regulatory nucleosome which might bind upstream of the *tra* operon promoter, controlling expression, possibly acting as such a repressor.

Histone-like proteins are a group of small, host-encoded DNA-binding proteins, which are common components of nucleosomal complexes, an obvious example being IHF, which is an essential component of the F relaxosome (Nelson *et al.*, 1995). Furthermore, histone-like proteins have emerged in recent years as common effectors of growth-phase dependent control in many systems. Recent work has identified two histone-like proteins, IHF and Fis, which repress *tra* operon expression as cells enter stationary phase. Both of these proteins appear to bind upstream of the *tra* operon. The presence of putative binding sites for TraJ and SfrA suggests potential interactions between these proteins serve to regulate expression of the *tra* operon (Lynch and Lin, 1996; Taki *et al.*, 1998). A putative DNA gyrase cleavage site was also identified upstream of $P_Y$, which is particularly interesting given that $P_Y$ has previously been shown to be highly dependent on supercoiling of the region (Gaudin and Silverman, 1993). Given the homeostatic control system observed in *E. coli* between Fis and DNA gyrase, it seems possible that the two might also interact to control the *tra* operon (Schneider *et al.*, 1999; Schneider *et al.*, 2000). However, further experimentation is required to determine if gyrase serves to alter transcription from $P_Y$ by controlling topology in the region.




As TraM levels had been observed to be heightened in stationary phase in *fis*⁻ mutant cells, sequence analysis was performed in the *oriT* region, upstream of *traM*, using the Fis binding site consensus sequence and sequence logo, in an attempt to identify any putative Fis binding sites (Finkel and Johnson, 1992; Hengen *et al.*, 1997).

Two putative sites were identified, along with, interestingly, adjacent putative DNA gyrase cleavage sites. Whether site-specific binding actually occurs at these sites is still unknown. The fluctuations in TraM levels may simply be reflective of changes in *traY* expression in the *fis*⁻ mutant cells.





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# *PLASMIDS AND BIOFILMS*

## ORAL PRESENTATIONS

**130** Natural conjugative plasmids induce bacterial biofilm development. Jean-Marc Ghigo

**131** *Escherichia coli* biofilm architecture induced by derepressed IncF plasmids. Ellen L. Zechner, Andreas Reisner, Mark A. Schembri, and Søren Molin

**Natural conjugative plasmids induce bacterial biofilm development**

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Horizontal gene transfer is recognized as a major source of evolution leading to change in the ecological character of bacterial species. Bacterial conjugation, which promotes the horizontal transfer of genetic material between donor and recipient cells by physical contact, is a phenomenon of fundamental evolutionary consequence. Whereas conjugation has been primarily studied in liquid, most natural bacterial populations are found associated with environmental surfaces in complex multispecies communities called biofilms. Biofilms are ideally suited to exchange of genetic material of various origins and recent reports demonstrated that bacterial conjugation occurs within biofilms. We investigated the direct contribution of conjugative plasmids themselves to the bacterial host capacity to form a biofilm. We showed that natural conjugative plasmids expressed factors that induced planktonic bacteria to form or enter biofilm communities, which favor the infectious transfer of the plasmid. This general connection between conjugation and biofilms suggests that medically relevant plasmid-bearing strains are more likely to form a biofilm. This may influence both the odds of biofilm-related infection risks and of conjugational spread of virulence factors.

# *ESCHERICHIA COLI* BIOFILM ARCHITECTURE INDUCED BY DEREPRESSED INCF PLASMIDS

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Bacteria utilize several mechanisms for adhering to and colonizing solid surfaces. Current models propose that surface adhesion of individuals followed by migration, replication and exopolymer production lead to the formation of bacterial biofilms. The prevalence and tenacity of biofilms is cause for persistent problems in medical and industrial settings. Accordingly, the factors governing biofilm formation and development receive increasing attention. A variety of extracellular molecules and surface organelles participate in biofilm development (1). In *E. coli*, flagella, type I pili and curli fimbriae are involved in attachment and adherence. Aggregative adhesive fimbriae encoded by large virulence pAA plasmids are important to biofilm formation in enteroaggregative *E. coli* as well (2). In *Pseudomonas*, flagella and type IV pili have been implicated (1). Recently, Ghigo (3) provided the first evidence that conjugative plasmids induce biofilm formation of different *E. coli* K12 laboratory strains in continuous flow cultures. Here we report the consequences of maintenance of IncF plasmids by *E. coli* strains on biofilm architecture and development. The strains were genetically tagged with variants of the green fluorescent protein. For this purpose, homologous recombination of linear double-stranded targeting DNA was mediated by the bacteriophage λ recombination functions. Monitoring of the development of flow chamber grown biofilms was facilitated by scanning confocal laser microscopy. Image analysis was performed by the software COMSTAT (4). Plasmid-free *E. coli* CSH26 formed thin (<10 μm), poorly differentiated biofilms in glucose minimal medium. In contrast, when this strain harbored derepressed IncF plasmids, thick, highly structured biofilms developed. These communities exhibited the large, vertically stacked (>100 μm) mushroom-shaped microcolonies interspersed with medium-filled channels that are classically associated with differentiated biofilms. The same biofilm phenotype was observed with other *E. coli* strains harboring these plasmids. Neither Ag43, type I fimbriae nor flagella were required for development of this biofilm morphology in *E. coli* MG1655. Expression of IncF conjugative pili was apparently sufficient to enable *E. coli* to associate in complex heterogeneous structures independently of these cell surface components. Furthermore, the AI-2 quorum sensing system of *E. coli* K-12 was also dispensable since the presence of plasmids in the domesticated laboratory strain DH5alpha, which harbors a frameshift mutation in the autoinducer *luxS* gene, promoted comparable biofilm architecture in flow cells. Variation in the biofilm phenotypes exhibited by CSH26 carrying plasmids with *tra* mutations indicate that elaboration of the conjugation machinery and not just pilin expression per se is important to cell-cell interactions in this context. Maintenance of the transfer repressed wild type R1 plasmid

did not contribute to biofilm development compared to the plasmid free state unless conjugation was supported by coinoculation of the flow chambers with donors and a suitable recipient. Thus, the capacity of CSH26[R1] to form microcolonies under the conditions of this study required conjugative spread of the plasmid. These findings support the view that biofilm formation is multifactorial and complex. The presence of IncF conjugative plasmids in a bacterial population stimulates the three dimensional development of a biofilm. Cells expressing conjugative pili predominate in the vertical outgrowth of the biofilm. Under conditions favorable to conjugation, the presence of a repressed plasmid such as R1, results in the elaboration of biofilm structures similar to those developed with its derepressed counterpart R1*drd*19. Plasmid expression of biofilm-promoting factors may offer an ecological advantage to their hosts.

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# *PLASMID EVOLUTION AND DIVERSITY*

## ORAL PRESENTATIONS

**134** (Conjugative) genomic islands as the fifth columnists of bacterial adaptation: Evolutionary lessons from the sequence analysis of R391. A. Mark Osborn

**137** Cooperative interactions in the regulation of replication, maintenance and transfer of IncP-1 plasmid. Christopher M Thomas, Malgorzata Zatyka, Sue Manzoor, Katarzyna Kazimierczak, Maciek Lukaszewicz and Lewis H. Bingle

**139** Interaction between two related IncQ-like plasmids isolated from bacteria that grow in the same environment. Douglas E. Rawlings, Murray, N. Gardner, Leonardo J. van Zyl and Shelly M. Deane

**141** Gene conversion leads to concerted evolution between members of a plasmidic multigene family in *Rhizobium*. Gustavo Santoyo, Edgardo Sepúlveda and David Romero

**143** Colicin K synthesis is posttranscriptionally regulated by the stress alarmone ppGpp and is expressed in up to 3 % of the colicinogenic population. Irena Kuhar, Janez Mulec, Zdravko Podlesek, Bart J.A.M. Jordi, Jos P.M. van Putten and Darja Žgur-Bertok

**145** Comparison of sequences of five homologous theta replicons from *B. megaterium* QM B1551 and progress in complete sequencing of the seven plasmid array. Patricia S. Vary, M. Kunnimalaiyaan, M. S. Scholle

**147** Restriction modification systems as selfish mobile genetic elements maintaining and rearranging the genome. Ichizo Kobayashi

## POSTER PRESENTATION

**149** In *Rhizobium etli*, a specific recombination system allows cointegration of the symbiotic plasmid with the conjugative plasmid p42a. Susana Brom, Lourdes Girard, Cristina Tun-Garrido, and David Romero

# (CONJUGATIVE) GENOMIC ISLANDS AS THE FIFTH COLUMNISTS OF BACTERIAL ADAPTATION: EVOLUTIONARY LESSONS FROM THE SEQUENCE ANALYSIS OF R391

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The classical paradigm of mobile genetic elements (MGEs) involved in horizontal gene transfer is the conjugative plasmid. The extrachromosomal nature of plasmids has readily enabled both determination of their presence, and subsequently the extensive characterisation of a number of important medically and environmentally important examples. However, in recent years the discovery of an increasing array of other conjugative MGEs, for example conjugative transposons (CTns) and conjugative genomic islands, suggests a hitherto undiscovered wealth of potential vectors and mechanisms for the exchange of genes between bacteria. These elements as a consequence of their integration into the bacterial chromosome, have led a covert lifestyle typically providing phenotypic evidence of their existence, for example in the transfer of antibiotic resistance, or hydrocarbon degradative functions, but with no readily visible signature ie. cccDNA within the cell. Finally though, these fifth columnists of the horizontal gene pool are yielding their secrets, and enabling us to characterise them in terms of their molecular mechanisms, and the evolutionary relationships of these elements to both plasmids and bacteriophages.

Genomic islands are now recognised as important contributors to bacterial adaptation and evolution having first been identified as clusters of virulence-associated genes in a number of pathogenic bacterial species and subsequently designated as pathogenicity islands (PAIs), with more recently characterised examples carrying symbiotic (Sym) or degradative functions (Sullivan & Ronson, 1998; van der Meer *et al.*, 2001). Typically, genomic islands range from 10 - 500 kb in size and often differ in G+C content and codon usage from the surrounding chromosomal DNA. They are regularly integrated into, or near, tRNA genes and flanked by short direct repeats resembling phage attachment sites. Phage-related integrases or remnants of such genes are typically found and enable these elements to be integrated into, and excised from, the bacterial chromosome. Additionally, an increasing number of genomic islands are being shown to possess the ability to transfer by conjugation.

This presentation will focus on the MGE R391 which for thirty years has represented an excellent example of this covert existence. R391 and the related "IncJ elements" have led a nomadic existence in terms of their classification since their discovery. Having originally been described as transmissible resistance factors (Coetzee *et al.*, 1972) and initially assumed to be plasmids, they were re-classified on the basis of their RecA-independent transfer and chromosomal integration, as CTns (Murphy and Pembroke, 1995). More recently, the demonstration of the chromosomal integration of R391 employing a phage-like integration system closely related to that of the SXT element from *Vibrio cholerae* led to a proposal that they should be included in the newly proposed group of CONSTINs (conjugative, self-transmitting, integrating elements) (Hochut *et al.*, 2001).

Subsequently the complete nucleotide sequence (89kb) of R391 has been determined (Böltner *et al.*, submitted), and has revealed that R391 is a mosaic comprising

a phage lambda-related integrase, and conjugative transfer functions related to those of the IncHI plasmid R27 from *Salmonella typhi*; together with an assortment of transposons and IS elements. From this analysis it is readily apparent that R391 shares the classical characteristics of a genomic island (albeit a conjugative element), in terms of its mode and type of chromosomal integration, though interestingly integration of R391 is unusual in that the preferred attachment site is in the *prfC* gene, as opposed to tRNA genes. On this basis, R391 has been classified as a conjugative genomic island.

However, in proposing this nomenclature it is necessary to open a broader debate on the classification and evolution of genomic islands and CTns as a whole. CTns represent a diverse group of self-transmissible elements, normally integrated into the chromosome, but capable of excision and integration; and transfer by conjugation. Whilst exhibiting a transposon-like phenotype in terms of their ability to excise from and integrate into the chromosome, the molecular mechanism clearly differs from that of "classical" transposons such as Tn*5* and Tn*10*, and is instead related to lambdoid phages (Salyers *et al.,* 1995). With conjugative transfer functions related to plasmids this makes them hybrid elements comprising phage integration and plasmid conjugation functions. Thus the designation of such elements as transposons, albeit conjugative, is perhaps arguable and the term transposon might best be reserved for transposons possessing a transposase gene. When such CTns are compared with the large group of genomic islands similarly employing a phage-like integration mechanism together with, in some cases, conjugative transfer functions related to those from plasmids, it becomes apparent that there is no clear differentiation based on molecular evidence to distinguish between CTns, CONSTINs and genomic islands. On this basis, to initiate a debate in the research community on MGE nomenclature it is suggested that genomic islands be used as a common denominator to classify R391 and related elements, and also those elements previously termed conjugative transposons.

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**Cooperative interactions in the regulation of replication, maintenance and transfer of IncP-1 plasmids**

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Regulation of gene expression on the IncP-1 genome is governed by a complex set of circuits that can be used as model for the layers of regulation observed on any bacterial genome. Regulation builds from local circuits controlling just one operon (for example the *kfrA* or *traK* autoregulatory circuits) to intermediate levels of complexity like the *korC* regulon controlling two or three operons, to global circuits like that governed by *korB* whose product binds at 12 sites on the plasmid genome and regulates at least six promoters (1). KorB binding sites exhibit a hierarchy of binding strength and are located in a variety of positions relative to promoters, immediately upstream (class I), at moderate distance upstream or downstream (class II) and at a great distance from any known promoter (2). Dissection of KorB has defined regions involved in dimerisation, DNA recognition, operator selectivity, repression of transcription (possibly through direct interaction with RNA polymerase), and interaction with other IncP-1 encoded proteins – IncC, KorA and TrbA (3). In particular we have shown that KorA and TrbA interact cooperatively with KorB when these proteins bind adjacent to each other (4,5). The question that we wished to address is the reason for these multiple regulators and their significance in vivo. We hypothesised that the circuits are set so that plasmid activity is maximised while the burden to the host is minimised. To investigate this we wished to determine the consequences of mutating these circuits, bearing in mind that in some complex systems there is a phenomenon known as robustness that means that the regulatory system is buffered against small changes in any parameter. We chose the circuits governing plasmid transfer to investigate this, and the action of KorB and TrbA at the trbB promoter in particular because the trb operon encodes the proteins of the mating pair apparatus. We first defined the operator for TrbA by point mutation analysis, since we had not previously been able to identify an obvious consensus palindrome at sites where we thought that TrbA acted (6). This showed that TrbA acts at *trbBp*, *traGp*, *traJp* and *traKp*, but that at none of these sites is its operator maximised for TrbA-mediated repression – a single base change could increase repression about 50%. When we introduced this change into the IncP-1 genome we observed greater that 100-fold decrease in plasmid transfer frequency. We showed that this decrease can not be explained by the effect of TrbA alone, but is dependent on the cooperativity of KorB and TrbA amplifying the effect of the point mutation. Indeed, the results imply that normally TrbA is set at a level that it only functions through cooperative interactions with KorB and would have little effect if present alone. Thus the cooperative circuits may provide a very efficient way of regulating expression at relatively low repressor concentrations, and over small changes in concentration.

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INTERACTION BETWEEN TWO RELATED INCQ-LIKE PLASMIDS ISOLATED FROM BACTERIA THAT GROW IN THE SAME ENVIRONMENT.

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IncQ and IncQ-like plasmids are characterized by their relatively small size (typically 6 - 15 kb) as well as their abilities to replicate in a very broad range of bacteria and to be efficiently mobilized by self-transmissible plasmids of the IncP group. This combination of properties has resulted in these plasmids being highly promiscuous. Plasmids that contain IncQ-like replicons may be divided into two major groups, depending on whether their mobilization genes are IncQ or IncP plasmid-related. We have isolated two IncQ-like plasmids from bacteria that grow in a similar, highly specialised ecological niche. Plasmid pTF-FC2 is 12.2 kb in size and was isolated from *Acidithiobacillus ferrooxidans*, while plasmid pTC-F14 is 14 kb and was isolated about 15 years later from *Acidithiobacillus caldus*. Both bacteria are aerobic, chemolithotrophic, sulfur-oxidizing, acidiphiles, but whereas *At. caldus* is moderately thermophilic (25-55°C), *At. ferrooxidans* is mesophilic (15-40°C) and can also oxidize iron. As both types of bacteria are frequently isolated from the same specialised environment, and as both IncQ-like plasmids are highly promiscuous, it is not unlikely that the two IncQ-like plasmids may encounter each other in the same host cell. Plasmids pTF-FC2 and pTC-F14 are clearly related with their RepA, RepB, and RepC replication proteins, their PasA, PasB toxin-antitoxin plasmid addiction proteins and their MobA, MobB, MobC, MobD and MobE mobilization proteins sharing 74 to 81, 72 to 81 and 21 to 78% amino acid sequence identity respectively. The two plasmids must therefore have originated from the same ancestor.

Based on the assumption that the plasmids are likely to encounter each other in their natural environment, we placed both plasmids into the same *E. coli* host cell and tested to what extent their replicons, toxin-antitoxin and plasmid mobilization systems were able to complement or compete with each other. It was hoped that this would give some insight into the pressures for each system to evolve to complete functional independence or whether they would retain a level of complementation.

Although most features of the replicons were highly conserved, the plasmids were found to have evolved to the point where they are compatible with each other. Neither set of plasmid iterons (which are the strongest incompatibility determinants) when cloned into a high copy number plasmid, could displace the other plasmid. Furthermore, neither set of RepA (helicase), RepB (primase) or RepC (DNA-binding) proteins from one plasmid could complement the *oriV* of the other. More detailed cross-complementation studies indicated that the RepC-DNA binding protein of each plasmid was *oriV*-specific but that the RepA and RepB proteins were able to cross-complement the related plasmid. The replicons of plasmids pTF-FC2 and pTC-F14 have therefore evolved sufficiently for them to be able to survive in the same host and during the process of evolution, the *oriV* regions and RepC proteins have become plasmid-specific. The phenomenon of

incompatibility between similar replicons has presumably placed pressure on the two replicons to evolve to the point of non-competitiveness and the finding that this had occurred was not unexpected.

In contrast to replication, plasmid mobilization is not understood to result in incompatibility within a host cell. Therefore the two mobilization systems may not directly compete with each other and the advantages or disadvantages for divergent evolution of the elements associated with plasmid mobilization are therefore less predictable. We attempted to address the questions: Have the two sets of mobilization genes diverged sufficiently for them not to complement the mobilization activity of each other? Has one of the plasmids evolved a mobilization system that would allow it to dominate the horizontal transfer process, thereby giving it a selective advantage in preference to the other? Both plasmids were found to have IncP-related mobilization genes. When using an RP4 helper system, the mobilization system of pTF-FC2 was more than 1000-fold more efficient at plasmid transfer between *E. coli* cells than pTC-F14. If both plasmids were placed in the same *E. coli* host, the presence of pTF-FC2 raised the mobilization frequency of pTC-F14 by about 50-fold. By adding parts of the mobilization region of pTF-FC2, it was found that the presence of the region containing the *mobC*, *mobD* and *mobE* genes resulted in increased mobilization. Other comparative aspects of plasmid mobilization will be described, such as the ability of the mobilization genes of pTF-FC2 to mobilize the *oriT* of pTC-F14, but not vice versa. In summary, although the mobilization of pTF-FC2 was much more efficient than that of pTC-F14, no evidence of plasmid competition during the mobilization process was evident. Rather, the opposite occurred, i.e. the frequency of horizontal transfer of the less efficient plasmid was assisted by the presence of the other.

With respect to evolution and interactions between plasmid toxin-antitoxin addiction systems the question asked is, whether there is an advantage for two compatible plasmids to have evolved cross-reacting or competing toxin-antitoxin systems? Antitoxins have at least two functions, neutralization of the toxin and autoregulation of the toxin-antitoxin operon. We therefore examined whether the antitoxins could interact to neutralize the heterologous toxin and also 'autoregulate' the heterologous operon. Should the two toxin-antitoxin systems not interact with each other, then should one plasmid be lost, the host cells may die and both plasmids would be eliminated. In contrast, should the toxin-antitoxin systems cross-react with each other, then the toxin-antitoxin systems would not serve as a plasmid stability system for the individual plasmids when in the presence of each other. However, if either plasmid was in a host on its own, the system should enhance plasmid stability. We found that the three-component PasA, PasB and PasC system of pTF-FC2 was a more efficient plasmid stability system than the two-component PasA and PasB system of pTC-F14. However, the PasA antitoxin of either plasmid was able to neutralize the heterologous toxin and likewise the antitoxins were able to 'autoregulate' the heterologous *pas* operons.

Based on the evidence obtained from these two related plasmids, the evolutionary pressure to diverge appears to have been greater on the plasmid replicons than on the toxin-antitoxin or mobilization systems.

# GENE CONVERSION LEADS TO CONCERTED EVOLUTION BETWEEN MEMBERS OF A PLASMIDIC MULTIGENE FAMILY IN *Rhizobium*.

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Rhizobial genomes are good models for the study of genomic plasticity, given the possession of plasmids of large size (representing over one third of the genome) and high levels of repeated sequences (either ISs or repeated genes) which may represent from 10 to 18% of the genome. In most *Rhizobium* species, genes responsible for nitrogen-fixing symbioses with leguminous plants are concentrated on a single plasmid, the so-called symbiotic plasmid or pSym.

For *R. etli*, the symbiont of the common bean plant, we have shown that the pSym (371 Kb) displays significant recombinational plasticity. Homologous recombination between the three members of the nitrogenase multigene family promote a variety of high frequency rearrangement events, deletions ($10^{-4}$), amplifications ($10^{-3}$), replicon fusions ($10^{-4}$), translocations ($10^{-5}$) and inversions ($10^{-7}$). These rearrangements may have important consequences for symbiotic proficiency; deletions of the symbiotic zone eliminate the ability to interact with beans, but duplication of this region enhances the nitrogen-fixing ability (Romero et al., 1997).

The repeats participating in this plasticity constitute the *nif* multigenic family. The *nifH* gene is identical between the three repeats; high identity is also seen among the *nifD* and *nifK* genes located in two of the repeats. The identity between these cannot be explained by functional compromise for the corresponding genes, since changes are absent even in the third position of codons. A more plausible alternative is the operation of a gene conversion process among the members of this prokaryotic multigene family.

*Gene conversion* is a recombinational mechanism that leads to non-reciprocal transfer of sequence information between homologous or homeologous genes. It arises through the repair of double strand breaks or heteroduplex DNA generated during recombination. Since gene conversion is not necessarily linked with crossover formation, it allows the transfer of sequence information between repetitions without formation of rearrangements. This process may lead to the maintenance of sequence homogeneity between members of a dispersed multigene family within a strain, but allowing variation between strains, a pattern known as *concerted evolution.*

Previous work using specific genetic constructs, has shown the operation of gene conversion between members of the *nif* multigene family (Rodríguez and Romero, 1998). Gene conversion events between the repetitions occur at a rate 100-fold higher than spontaneous mutation. Frequencies of gene conversion were not affected by the disposition of the interacting repeats (either in cis or in trans).

An important parameter to be analyzed is the mean length of the transferred segment. The knowledge of this parameter, will allow us to predict more accurately the dynamics of the homogenization process in a plasmidic multigene family. To that end, we have modified the *nifH* gene by introducing single-base pair changes every 100 bases along the gene. These changes create also specific restriction sites. Use of this modified

gene as a donor in gene conversion events, allowed us to infer the length of the transferred segment, by analyzing which sites are gained or lost. Our results show a tight linkage between recombination and gene conversion. Out of fifty recombination events analyzed, forty nine displayed a gene conversion event close to the crossover site. More than 75% of the gene conversion events analyzed display the transfer of continuous segments, with a mean length of 550 bp. The remaining events showed a "patchy" distribution, where a segment undergoing crossover is flanked by small segments (about 150 bp) displaying gene conversion. For both types of events, transfer of information is strongly biased toward the acquisition of restriction sites, rather than its loss.

This work was further extended by studying, using a population genetics approach, if gene conversion generates a pattern of concerted evolution. To that end, we have obtained, by specific PCR amplification, each of the nitrogenase repetitions of ten different *R. etli* strains from variant geographical locations. Nucleotide sequence of each *nifH* gene was obtained and analyzed. The cladograms obtained revealed that for seven of the strains the sequence of the repeats was identical both within and between the strains, suggesting a recent origin. However, for three strains, the sequence of the repeats was highly similar within a strain but less similar between strains. These results indicate that gene conversion is generating a pattern of concerted evolution between members of the *nif* multigenic family.

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# COLICIN K SYNTHESIS IS POSTTRANSCRIPTIONALLY REGULATED BY THE STRESS ALARMONE ppGpp AND IS EXPRESSED IN UP TO 3 % OF THE COLICINOGENIC POPULATION

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Colicins are plasmid encoded bacteriocins, synthesized by, and active against *Escherichia coli* cells and sometimes cells of closely related species such as *Shigella* and *Salmonella*. Colicin producing strains occur with high frequency among natural isolates and they have been implicated in intraspecies population dynamics. Besides acting to eliminate competitors and providing nutrients, colicin synthesis is also a programmed cell death mechanism. Colicin synthesis results in lysis of the sensitive cell and the producing cell as well. Further, loss of the colicin encoding plasmid results in loss of immunity, providing a mechanism for plasmid maintenance.

Colicin K belongs to the group of ColE1 pore-forming colicins which destroy the electrochemical potential of the cytoplasmic membrane. Production of the bacteriocin is encoded by three genes, *cka* encoding the colicin, *cki* encoding immunity and *ckl* encoding lysis (1).

Synthesis of colicin K is characteristically regulated by the SOS response via the LexA repressor. Additionally, synthesis is growth phase dependent and induced by nutrient depletion due to an increase in ppGpp (2). Comparison of specific mRNA levels before and after nutrient starvation demonstrated that ppGpp acts posttranscriptionally. Synonymous replacement of rare codons by more frequently used ones at the 5' end of the *cka* mRNA, as well as increasing the availability of the rare leu tRNA, abolished ppGpp mediated regulation. ppGpp thus regulates colicin K synthesis via a novel post-transcriptional mechanism that is based on rare codon usage and variable cognate tRNA availability (3).

Since colicins are released semi-specifically by cell lysis, genes of the pore forming colicins should be expressed differentially so that only a part of the population expresses the activity and lysis genes. To observe expression of the colicin K activity and immunity protein genes at the single cell level through the growth cycle, transcription fusions of the *cka* and *cki* promoters and the promoterless *gfp* were prepared on the natural colicin K encoding plasmid pColK-K235. Fluorescence microscopy revealed that the *cka* gene is expressed in only 3 % of the bacterial population upon induction by nutrient starvation while the immunity gene *cki* is expressed in the large majority of the cells. Mitomycin C induction resulted in transcription activation of the *cka* gene in almost all of the analysed cells demonstrating that differential expression is primarily established at the level of transcription (4).

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# COMPARISON OF SEQUENCES OF FIVE HOMOLOGOUS THETA REPLICONS FROM *B. MEGATERIUM* QM B1551 AND PROGRESS IN COMPLETE SEQUENCING OF THE SEVEN PLASMID ARRAY.

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*B. megaterium* QM B1551 contains a seven plasmid array. The plasmids are pBM100 (5.4 kb), pBM200 (9.1 kb) pBM300 (26 kb), pBM400 (55 kb), pBM500 (71 kb), pBM600 (108 kb) and pBM700 (165 kb) (1, 3). The two smallest plasmids replicate by rolling circle mechanism, while the five large plasmids replicate by a theta mechanism. A probe from one replicon hybridizes with all five plasmids (2). The four smaller plasmids (5.4-55 kb) have been isolated in a strain by themselves. Four of the five compatible theta replicons of *B. megaterium* QM B1551 have now been sequenced and the fifth is being sequenced. The RepM proteins are 80-89% identical and their ORFs contain a set of three or four 12 bp tandem repeats (iterons) with very close homology. Four of the five replicons also have an ORFB downstream involved in plasmid stability. Two replicons contain an ORFC, probably in the same operon, but with no detectable function in replication. In contrast, pBM300 contains an ORF2 downstream that has no homology to ORFB, yet is required for stability, and no ORFC. None of the above proteins has similarity to other proteins in the databases, so that the five replicons define a new, very homologous, yet compatible family. Several interesting genes are also upstream of the replicons including a sigma H gene, the initial sporulation specific sigma factor (pBM500), a complete rRNA operon (pBM400), *tra*, *mob* and *ori T* (pBM300). Genes with high similarity to genes on the *B. subtilis* genome, including a possible copper export gene, are also present. There is an area upstream from the RepM genes in two of the plasmids that has large inverted repeats (79 and 80 bp flanking 640 bp, but containing no ORF). Also, downstream from pBM700 there is a very large (180-200) bp direct repeat.

In addition, our laboratory is in the process of completely sequencing all seven plasmids. The 5.4, 9.1, 26 and 55 kb plasmids are completed (Zhou, Baisa, Scholle, Kunnimalaiyaan, to be submitted). We have considerable sequence from the 108 kb plasmid and are in the process of sequencing the 71 kb and 165 kb plasmids. Genes on these plasmids include several with similarity to genes of *Streptococcus, Staphylococcus* and *B. anthracis* plasmids. There are also antibiotic resistance genes, transposases and unusual degradative enzyme genes, such as a styrene monooxygenase gene. It is postulated that there is considerable plasmid exchange among the Gram positive bacteria.

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# RESTRICTION MODIFICATION SYSTEMS AS SELFISH MOBILE GENETIC ELEMENTS MAINTAINING AND REARRANGING THE GENOME

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A restriction enzyme gene is often linked to a modification methylase gene whose role is to protect the recognition site from breakage by the restriction enzyme. Attempts to eliminate some of these restriction-modification gene complexes from bacterial cells lead to cell death through restriction breakage in the genome (Naito et al. Science 1995). Such post-segregational cell killing was observed when a restriction-modification gene complex was challenged by a competitor genetic element and likely has competitive advantage.

Comparison of closely related bacterial genomes revealed linkage of genome polymorphisms, such as insertion and inversion, with restriction-modification gene complexes. Restriction site avoidance in bacterial genomes and other observations provide further support for our hypothesis that some restriction-modification gene complexes behave as selfish mobile elements that have attacked and shaped the genomes (Kobayashi, Nucleic Acids Research, 2001).

Indeed our attempts to eliminate a restriction-modification gene complex from a cell led to various genome rearrangements in the laboratory --- genome-wide duplication and inversion events (Handa et al. Molecular Microbioloy 2001), intra-genomic movement of a restriction-modification gene complex (Ohashi, Ui-Mizutani, Kobayashi, unpublished), and tandem amplification of a restriction-modification gene complex (Sadykov, Asami, Itaya, Niki, Tanokura, Kobayashi, unpublished). These genome rearrangements are likely caused and selected by the restriction attacks.

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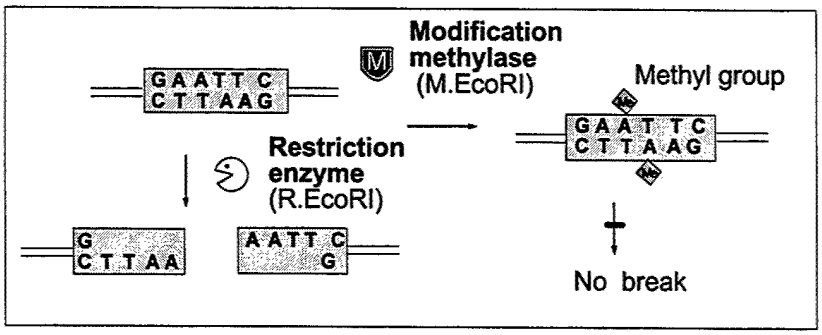
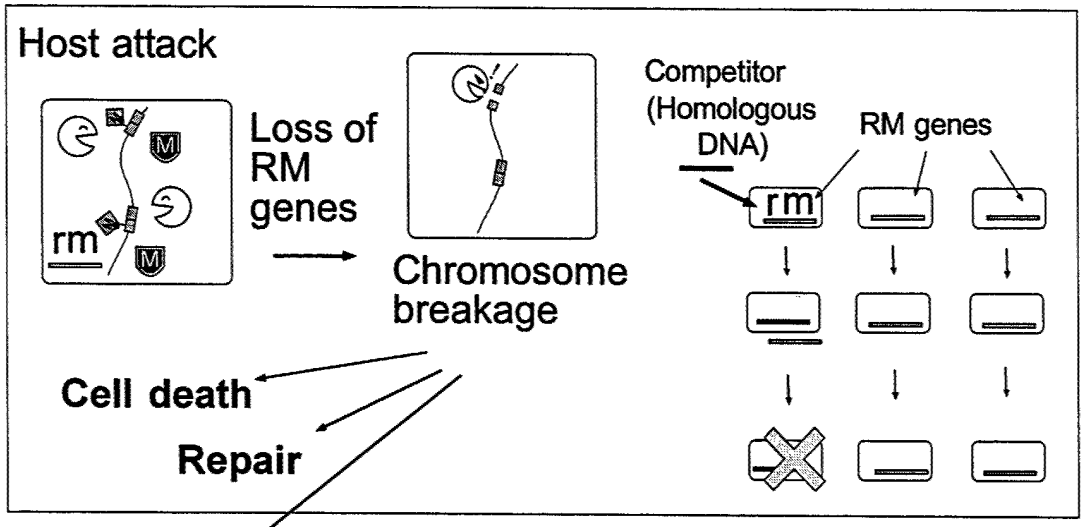



-- Destroys non-self (invader & host cell)

-- Moves between genomes

as viruses and transposons




Genome rearrangements

**Amplification** rm rm rm rm rm rm

**Transposition,inversion,duplication**

Genome evolution

# IN *Rhizobium etli*, A SPECIFIC RECOMBINATION SYSTEM ALLOWS COINTEGRATION OF THE SYMBIOTIC PLASMID WITH THE CONJUGATIVE PLASMID p42a.

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*Rhizobium etli* is able to establish a symbiotic relationship with the roots of *Phaseolus vulgaris* (beans). Most of the bacterial genes required for this interaction, are encoded in a plasmid (symbiotic plasmid or pSym). Additionally, *Rhizobium* strains usually contain other extrachromosomal elements. The *R. etli* type strain, CFN42, contains six plasmids (p42a to p42f), with sizes ranging from 150 to 600 kb. p42d (390 kb) corresponds to the pSym. A singular characteristic of the rhizobial genome, is its high content of reiterated sequences. These reiterations are present in the chromosome, in the different plasmids, or shared among different replicons.

We have shown that conjugative transfer of the pSym of strain CFN42 occurs at a frequency of $10^{-6}$, and depends on the presence of the self-transmissible plasmid, p42a, whose transfer frequency is in the range of $10^{-3}$. (Brom et al., 2000 Plasmid 44:34-43).

When transconjugants for pSym transfer are selected, 90% also inherit p42a. Analysis of the other 10% indicated that the pSym present in these transconjugants are recombinant plasmids, which have acquired segments of p42a, and lost DNA of the pSym. The p42a DNA present in the recombinant plasmids includes the *oriV*, and the *tra* region, consequently these plasmids are self-transmissible, and compatible with the wild-type pSym. Analysis of 10 different recombinant pSyms showed that most of them (7/10), share one border of the p42a-pSym junction. The sequence analysis of this region, showed the presence of a 50 bp segment that is 90% identical among the pSym and p42a. This segment includes a 23 bp inverted repeat. An ORF encoding a possible integrase-like protein was found near the homologous region, on p42a.

Taking these results into account, we propose that conjugative transfer of the pSym initiates with the formation of a pSym-p42a cointegrate, through specific recombination in the homologous zone described above, dependent on the integrase- like protein; the cointegrate is able to perform conjugative transfer. In most of the transconjugants (90%), the cointegrate utilizes the same specific recombination mechanism to regenerate the wild-type plasmids. In the other 10%, the cointegrate utilizes some other of the repeated sequences shared among the pSym and p42a, generating the recombinant plasmids.

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# *PLASMID ECOLOGY*

## ORAL PRESENTATIONS

**151** Marine plasmids: Insights from diversity studies and sequencing. Patricia Sobecky, Heath J. Mills, Keri Beeson and Marisa Cook

**153** IncP-1beta plasmids and chloaromatic degrading beta-proteobacteria, from alpha to omega. Eva Top, W. Dejonghe, N. Boon, W. Verstraete, J. Goris, and P. de Vos

**155** Determining the ecological mechanisms of plasmid adaptation to the niche in plant associated pseudomonad populations. Sarah L. Turner and Mark J.Bailey

# MARINE PLASMIDS: INSIGHTS FROM DIVERSITY STUDIES AND SEQUENCING

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Considerable knowledge has been gained from the intensive study of a relatively limited group of bacterial plasmids. Recent efforts have begun to focus on the characterization of, at the molecular level, plasmid populations occurring in a wider range of aquatic and terrestrial habitats (e.g., 2-4). Such studies will provide greater knowledge on the ecology of plasmids and their contributions to the genetic plasticity of naturally occurring microbial communities.

We are applying several different approaches including *inc/rep* typing, RAPD-PCR analyses and whole plasmid sequencing to gain a better understanding of marine plasmids and their contributions to marine bacterial assemblage structure and function. To determine whether *inc/rep* probes derived from naturally occurring marine plasmids could be used to assess marine plasmid dissemination and diversity, a collection of functional replication-proficient fragments (repGA14, repGA33, repGA70, repSC1, repSD41, repSD121, repSD164, repSD172) were obtained from broad-host-range marine plasmids ranging in size from 5 kb to 85 kb (2, 5). These rep origins were isolated from marine sediment bacteria belonging to the $\alpha$ and $\gamma$ subclasses of the *Proteobacteria*. Several of the marine plasmids occurred at high frequency in the culturable bacterial fraction (i.e., repGA33 and repSD41). However, these plasmids lacked readily identifiable phenotypic traits. We have found that the marine plasmid replicons repSD41/repSD121 and repGA70, originally isolated from two different marine *Vibrio* sp., can be detected in plasmid-bearing marine bacterial isolates belonging to the genera *Pseudoalteromonas* and *Shewanella* cultivated from geographically disparate coastal sites (California and Georgia USA). In contrast, repGA33 and repSC1 obtained from *Shewanella* sp. appeared to be geographically restricted to coastal Georgia and South Carolina salt marsh ecosystems respectively (2).

In addition to the isolation of auto-replicative fragments from marine plasmids, we have developed a PCR-based method that facilitates the assessment of plasmid populations along temporal and spatial gradients. This technique facilitates the comparison and differentiation of marine plasmids based on the randomly amplified polymorphic DNA (RAPD) method (6, 7). Two oligonucleotide primers (DIAZO1 and MPRP2) were developed to facilitate genotypic typing of endogenously isolated marine plasmids by the RAPD-PCR technique. These primers proved to be more effective than twenty-one commercially available primers tested to generate RAPD-PCR patterns. The method was subsequently used to determine the molecular diversity of plasmids occurring in a key ecological group present in marine sediment ecosystems (1). Analysis of the RAPD-PCR patterns indicated as many as seventy-one different plasmid genotypes

occurring in diazotroph (N-fixing) bacterial assemblages within and between the four different salt marsh grass rhizoplane habitats investigated in this study (1).

Recently, plasmids from a marine *Microscilla* sp. (101-kb) and a marine *Micrococcus* sp. (50-kb) have been sequenced (8, 9). In collaboration with The Institute for Genome Research (TGIR), we have also undertaken whole plasmid sequencing to elucidate putative gene functions and gain a better understanding of their diversity and ecological role of selected marine plasmids has been conducted. A random shotgun method has been used to obtain DNA sequences from numerous 'cryptic' marine plasmids ranging in size from 30-kb to 100-kb isolated from *Vibrio* and *Shewanella* sp. Sequencing reveals numerous phage-related genes, putative colicins and transfer systems encoded on several of the plasmids (Mills, Sobecky and Eisen, unpublished). A comparative and systematic analysis of the marine plasmid sequences will be presented.

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# INCP-1BETA PLASMIDS AND CHLOAROMATIC DEGRADING BETA-PROTEOBACTERIA, FROM ALPHA TO OMEGA...

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Genes that code for the degradation of both naturally occurring and xenobiotic organic compounds are often located on plasmids, transposons or other transposable or integrative elements. The list of published reports of such mobile genetic elements (MGEs) is growing continuously as researchers continue to isolate and characterize new degrading bacteria and their corresponding degradative genes. There is also growing evidence that horizontal exchange of catabolic (degradative) genes among bacteria in microbial communities plays an important role in the evolution of catabolic pathways. While these catabolic mobile elements shed more light on their role in the *in situ* construction of new pathways in bacterial communities, they may also be used as tools in bioremediation of polluted soils and waters. The first plasmids involved in degradation of organic pollutants have been described ca. 20 years ago. More than 10 years ago, an interesting observation about the incompatibility group of catabolic plasmids was made by Mergeay et al. (1990). Plasmids that encode degradation of naturally occurring compounds seem to belong to groups with a moderately broad host range, such as the incompatibility groups IncP2 and IncP9. In contrast, plasmids that encode degradation of compounds that are mainly man-made, such as several chloroaromatics, seem to often belong to the well known IncP-1 group of broad host range plasmids. This first observation has been confirmed by more recent studies of plasmids encoding degradation of chloroaromatics (Top et al., 2000). When these plasmids are characterized in more detail, they are even often found to belong to the same subgroup, i.e., IncP-1β. These plasmids are the most promiscuous self-transmissible plasmids characterized to date and have been initially identified as bearers of antibiotic resistance genes. It is hypothesized that their promiscuity has played a role in bacterial adaptation to newly introduced xenobiotics by recruiting catabolic genes or gene segments from different organisms, thereby yielding a new pathway in a suitable host (Beil et al., 1999). Previous studies and the ones described below have also pointed out that the majority of the bacterial hosts that carry these IncP-1β plasmids and express their degradative genes belong to the β-subgroup of the *Proteobacteria*, while a few γ- subgroup *Proteobacteria* are also found.

Our study of bacteria that are able to degrade chlorinated anilines entirely confirms the above-hypothesized correlation between IncP-1β plasmids and β-subgroup *Proteobacteria* that degrade chloroaromatics. First, we examined the diversity of plasmids in five bacterial isolates that are able to metabolize both aniline and 3-chloroaniline (3-CA) (Boon et al, 2001). The five strains belong to two different genera (*Comamonas* and *Delftia*) within the β-subgroup of the *Proteobacteria*, and possess a large plasmid of ca. 100 kb. Conjugation experiments revealed that four of the five

plasmids encode complete or partial degradation of aniline, while only one plasmid could transfer the ability to deaminate and dechlorinated 3-CA. None of the plasmids seemed to encode total 3-CA degradation. The four plasmids belong to the IncP-1 incompatibility group, and based on PCR amplification and hybridization with *korA* primers and probes, three of them seem to be IncP-1β plasmids. A following study extended the isolation and characterization of 3-CA and 3,4-dichloroaniline (3,4-DCA) degrading bacteria (Dejonghe et al., 2002). Amongst the 3-CA and 3,4-DCA degraders isolated from three different soils, two belong to the γ-subgroup and seven to the β-subgroup of the *Proteobacteria*. Interestingly, all isolates contained again an IncP-1β plasmid, based on their *trfA2* sequences. While all plasmids, except one, encode total degradation of 3-CA, no plasmids were found to encode 3,4-DCA degradation. Since chloroanilines are formed in nature during the degradation of several herbicides, such as the phenylurea herbicide linuron, we also isolated chloroaniline degrading bacteria from such herbicide treated soils after enrichment for linuron degradation. From such a linuron degrading consortium, two 3-CA and 3,4-DCA degrading strains (*C. testosteroni and D. acidovorans*) were isolated. Conjugation experiments showed that the complete degradation pathway of these two compounds in these β-*Proteobacterium* is encoded by the same plasmid, pWDL7, the first plasmid known to encode complete 3,4-DCA degradation. Based on its *trfA2* sequence, it is again an IncP-1β plasmid. Hypotheses for these striking and consistent correlations between the degradation of chloroaromatics, IncP-1β plasmids and β-subgroup *Proteobacteria* will be discussed.

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## *DETERMINING THE ECOLOGICAL MECHANISMS OF PLASMID ADAPTATION TO THE NICHE IN PLANT ASSOCIATED PSEUDOMONAD POPULATIONS.*

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Studies of the genetics of natural populations of bacteria have revealed the importance of the horizontal gene pool in the generation, replication and dissemination of the genes associated with host fitness. To understand the evolutionary role of plasmids we must develop a more complete knowledge of their phylogeny and distribution to determine the ecological relevance of horizontal gene transfer. Advances in the study of bacterial genomics demonstrate the central role of horizontal gene transfer in bacterial evolution, and the description of gene clusters associated with adaptive phenotypes has resulted in the concept of fitness islands which are found on both chromosomes and plasmids. Sequence and IVET (in vivo expression technology) of mega plasmid pQBR103 (conjugative, mercury resistant, 330kbp), common to plant associated pseudomonad populations, revealed only limited homology to existing bacterial genome data bases. Some homology has been observed that are typical of mobile elements; for example RepC, UvrC, UvrD, TnpA and MerRTP, but not to known transfer loci and a unique origin of replication. Of the few homologues identified to chromosomal genes, one was to an oligribonuclease. By IVET a number of (novel) genes have been identified that are expressed from the plasmid only when host bacteria colonise plant surfaces. By combining molecular and ecological studies will it be possible to determine the role of the horizontal gene pool in bacterial population biology, and test the hypothesis that the HGP is a genetically partitioned unit of the genome which carries unique determinants infrequently associated with the chromosome, but essential for local adaptation and periodic survival of the host.

# *VIRULENCE/ANTIBIOTIC RESISTANCE PLASMIDS*

## ORAL PRESENTATIONS

**157** The pJM1 plasmid encodes iron transport and regulatory genes that are essential in microbial pathogenesis. Jorge H. Crosa

**159** Functional analysis of the Tn*4451/4453* family of clostridial mobilisable transposons. V. M. Adams, I. Lucet, P.A. Howarth, J. Kim, D. Lyras and J. I. Rood

**161** The virulence plasmid sequence of *Shigella flexneri*. Malabi M. Venkatesan, Marcia B. Goldberg, Debra J. Rose, Erik J. Grotbeck, Valerie Burland, Frederick R. Blattner

**162** Novel domain organization of AngM, a nonribosomal peptide synthetase essential for siderophore biosynthesis in *Vibrio anguillarum*. Manuela Di Lorenzo, Sophie Poppelaars, Maho Nagasawa, Marcelo E. Tolmasky and Jorge H. Crosa

## POSTER PRESENTATIONS

**164** Complete nucleotide sequence of pMAC, a plasmid from *Acinetobacter baumannii* 19606 that encodes organic peroxide resistance. C.W. Dorsey, A.P. Tomaras and L.A. Actis

**166** Variation of plasmids from glycopeptide resistant *Enterococcus faecium* isolated from pigs in Denmark. Henrik Hasman, Anette G. Villadsen and Frank M. Aarestrup

**167** Characterization of a large plasmid, encoding streptogramin resistant, from an *Enterococcus faecium* of animal origin. Lars B. Jensen and A.M. Hammerum

**168** Organization of the plasmid *CPE* locus in *Clostridium perfringens* type A strains. Kazuaki Miyamoto, Ganes Chakrabarti, Yoshiharu Morino, and Bruce A. McClane

**169** Molecular characterization of plasmids encoding extended-spectrum ß-lactamases (ESBLs) from nosocomial enterobacterial isolates. J. Silva, U.Garza-Ramos, and G. Sánchez

**THE pJM1 PLASMID ENCODES IRON TRANSPORT AND REGULATORY GENES THAT ARE ESSENTIAL IN MICROBIAL PATHOGENESIS.**

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The bacterial fish pathogen *Vibrio anguillarum*, a gram negative polarly flagellated comma-shaped rod, is responsible for both marine and fresh water fish epizootics throughout the world. *V. anguillarum* causes a highly fatal hemorrhagic septicemic disease in salmonids and other fishes including eels. The disease caused by *Vibrio anguillarum* in salmon shows striking similarities to the septicemic disease in humans caused by *V. vulnificus*.

The key feature that enables many pathogenic strains of *V. anguillarum* to survive within the vertebrate host in feral, as well as in intraperitoneal experimental infections, is the possession of a 65 kb virulence plasmid, pJM1. This plasmid provides the bacteria with an iron-sequestering system that is crucial in overcoming the non-specific defense mechanisms of the host. This system centers upon the synthesis of the 348 Da-siderophore anguibactin, an iron-scavenging compound, and subsequent transport of the ferric-anguibactin complex into the cell cytosol via the cognate transport system proteins FatA, B, C, and D. Anguibactin is produced by the virulent strains of this bacterium in the host and in any other environment in which iron is chelated and thus not readily available. The plasmid-encoded iron-transport system and siderophore biosynthetic genes are controlled by the concentration of available iron, via at least four plasmid-encoded regulators: two positive regulators, AngR (anguibactin system regulator) and TAF [transacting factor(s)]; and two negative regulators, antisense RNAα and RNAβ. RNAα is very stable at high iron concentrations and acts at posttranscriptional level in the repression of *fatB* and *fatA* expression while RNAβ, found under conditions of mild iron-limitation, acts on the attenuation of expression of the *angR* gene in the ITB operon. Repression of the ITB operon at the transcriptional level requires the chromosomally-encoded Fur protein. The promoter for the iron transport-biosynthesis operon (pITBO) was localized within a region ca. 300 base pairs upstream of *fatD* by primer extension and S1 mapping analysis. One of the other elements controlling expression of the ITB operon is AngR. Remarkably, this protein plays a role not only in the regulation of iron transport gene expression but also in anguibactin biosynthesis, and therefore virulence. The angR gene is encoded within a polycistronic message that includes the iron transport genes *fatDCBA*, and the *angT* gene, within the iron transport-biosynthesis operon (ITB operon). In addition, there is evidence that anguibactin itself enhances transcription of this operon, possibly independently of AngR and the TAF products. Recently, the TAF region was dissected into two subregions: TAFb, essential for anguibactin biosynthesis, and TAFr, associated with regulation of expression of the iron transport-biosynthesis operon. The ITB operon and other anguibactin biosynthetic genes located downstream are bracketed by the highly-related ISV-A1 and IASV-A2 insertion sequences, which are also highly related to the insertion sequences found flanking various thermostable direct hemolysin genes in *V. parahaemolyticus, Vibrio mimicus*, and non-O1 *V. cholerae*. This raises the possibility that some of these genes may have been acquired during evolution.

The enzymology of anguibactin biosynthesis is still under investigation, however predictions can be made based on our knowledge of the structure of this siderophore and

functions of potential biosynthetic genes inferred by homology studies. The TAFb region consists of an apparent composite transposon, surrounded by ISAV2 repeated sequences, containing an ORF which is essential for anguibactin biosynthesis. This ORF, was demonstrated to encode AngB, a 287 amino acid polypeptide that shows significant homology to the EntB protein of *E. coli* and the VibB protein of *V. cholerae*. AngB is essential for the biosynthesis of an anguibactin precursor, DHBA. The amino terminus of AngB possesses the isochorismate lyase activity (ICL) thereby explaining the need for this protein for the synthesis of DHBA. Analysis of mutations in the *angB* open reading frame provided evidence that in addition to *angB,* an overlapping gene, *angG,* exists at this locus and that it encodes three polypeptides which are in-frame to the carboxy-terminal end of the 33 kDa AngB. In addition to the DHBA-synthesis function in the amino terminus (ICL) there is at the carboxy terminus, an ArCP domain that is also present in the internal AngG polypeptides (132). This domain is where phosphopantetheinylation occurs at the serine residue. The P-Pant group acts as an acceptor of an activated aryl or amino acid group. ArCP domains intervene in assembly reactions during siderophore biosynthesis. By using site-directed mutagenesis a mutation at S248 was generated that leads to a complete abolishment of anguibactin production as compared to the isogenic control. Yet DHBA production in this mutant was unaffected, further demonstrating the separability of the ICL and ArCP activities.

Transposon insertions in the virulence plasmid identified another gene, *angM,* involved in anguibactin biosynthesis which is discussed in Ms. Di Lorenzo's presentation.

AngR is essential for both regulation as well as anguibactin biosynthesis. The AngR amino acid sequence identifies the domain organization as Cy-A-PCP. The 10 subdomains of the A domain, A1-A10, are conserved. The PCP and Cy/C domains of AngR may not be functional because an essential serine is replaced by alanine in the PCP domain, while the essential first aspartic acid is replaced by asparagine in the Cy/C subdomain, although the other 7 Cy subdomains are very conserved. Another gene, *angT,* located downstream of *angR* and part of the ITB operon is also essential in anguibactin biosynthesis, since its deletion leads to a 17-fold decrease in anguibactin production. AngT is a homolog of thioesterases and could play a role in the disengagement of the anguibactin molecule after biosynthesis ends.

The first two rings of the anguibactin structure are analogous to those in yersiniabactin (Ybt) and pyochelin (Pch), a phenolic-thiazoline moiety for iron chelation, but use dihydroxybenzoate rather than salicylate as monomeric precursor, so the anguibactin has a catechol rather than a phenol substitution pattern. The right-hand half of anguibactin differs from Pch and Ybt in that this siderophore is not a free COOH siderophore but rather has the dihydroxyphenylthiazolinyl (DHPT) acyl group in amide linkage to an N-hydroxy-histamine. Formation of this amide is a distinguishing characteristic of the anguibactin assembly line. The anguibactin cluster contains two genes that are dedicated to conversion of the readily available amino acid histidine to the N-hydroxyhistamine dedicated cosubstrate, a histidine decarboxylase, AngH, and an N-oxygenase, AngU. Experiments are currently being carried out to purify the anguibactin NRPSs and synthesize anguibactin in vitro.

# FUNCTIONAL ANALYSIS OF THE Tn*4451/4453* FAMILY OF CLOSTRIDIAL MOBILISABLE TRANSPOSONS

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The chloramphenicol resistance transposon Tn*4451* is located on a conjugative plasmid in *Clostridium perfringens* and the closely related element Tn*4453a* is found on the chromosome in *Clostridium difficile*. Tn*4451* and Tn*4453a* encode six very similar genes, *tnpX, tnpV, catP, tnpY, tnpZ* and *tnpW*. The *catP* gene confers chloramphenicol resistance and *tnpZ* encodes a transposon mobilisation protein. The *tnpV*, *tnpY* and *tnpW* genes do not have any known function.

TnpX is responsible for the excision and insertion of these transposons. The N-terminal TnpX domain has similarity to the resolvase family of site-specific recombinases but at 707 aa TnpX is three times the size of most resolvases. We have shown that transposition occurs by a resolvase-mediated site-specific recombination process that involves the formation of a circular intermediate. Based on computer analysis of TnpX we constructed three deletion derivatives that were truncated at the R356, K492 and E597 codons. These mutants were compared to the wild-type gene using *in vivo* excision and transposition assays. The results showed that although the *tnpXΔ598-707* derivative was functional, *tnpXΔ357-707* and *tnpXΔ493-707* did not confer excision. In transposition assays *tnpXΔ493-707* exhibited negative dominance over the wild-type *tnpX* gene.

The wild-type TnpX protein and the deletion derivatives were over-expressed, purified as C-terminal His-tagged proteins, and shown by gel filtration to be able to form dimers. Gel shift analysis showed that the TnpX protein bound very efficiently to DNA fragments that contained the ends of the integrated element or the joint of the circular intermediate. However, it bound several orders of magnitude less efficiently to its target sites. DNaseI footprinting showed that TnpX bound on both strands to a 35-45 bp region that encompassed the transposon ends. The TnpXΔ598-707 protein bound with similar affinity to the wild-type TnpX protein, in agreement with *in vivo* results, whereas the TnpXΔ357-707 and TnpXΔ493-707 proteins bound much less efficiently, if at all. These results imply that the negative dominance effects result from the formation of an inactive dimer containing both wild-type and mutant monomers.

To identify specific regions that are required for TnpX activity we developed a method for detecting *tnpX* mutants with altered function. After passage through the mutator strain XL1-Red, 22 random *tnpX* mutants were isolated and analysed. Most of the mutations were located in the N-terminal resolvase domain but four were located outside this region. Excision and transposition assays indicated that the 251-261 aa region was of functional significance. Analysis of the purified TnpX-E251K and TnpX-K260E proteins showed that although these proteins could bind to the specific DNA target their binding characteristics were aberrant and their binding affinities significantly reduced.

In summary, we have purified the large TnpX resolvase and shown that it binds to a DNA target site that is located at either end of the transposon or at the joint of the circular intermediate. We have shown that the C-terminal 110 amino acids are not essential for biological activity and have identified an internal region that either

represents the DNA binding domain or is required for the correct conformation of the DNA binding domain.

# THE VIRULENCE PLASMID SEQUENCE OF *SHIGELLA FLEXNERI*

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The complete sequence analysis of the 210 kb *Shigella flexneri* 5a virulence plasmid was determined. *Shigella* spp. continue to be a major health problem worldwide, causing an estimated 1 million deaths and 163 million cases of dysentery annually, predominantly in children younger than five years of age in developing countries. *Shigella* cause bacillary dysentery in humans by invading and replicating in epithelial cells of the colon, causing an intense inflammatory reaction, characterized by abscess formation and ulceration, which damages the colonic epithelium. Most work on the molecular pathogenesis of *Shigella* has been carried out in *S. flexneri* serotypes 2a and 5a. The entire complement of genes critical for invasion of epithelial cells is contained on a large 210 kb plasmid, termed the virulence plasmid or the invasion plasmid, which is present in all pathogenic strain. Known to be located on the virulence plasmid is a locus of genes (*ipa-mxi-spa*) that encode proteins involved in invasion of mammalian cells and which has homologs in *Salmonella*, *Yersinia*, enteropathogenic *E. coli*, the plant pathogens *Ralstonia solanacearum*, *Xanthomonas campestris,* and the flagellar assembly loci of *S. typhimurium*. In addition to those mentioned above, several other virulence plasmid proteins have been previously characterized Most of the known *Shigella* virulence determinants account for approximately 30-35% of the virulence plasmid. In the complete sequence of the virulence plasmid, 286 open reading frames (ORFs) were identified. An astonishing 153 (53%) of these were related to known and putative insertion sequence (IS) elements; no known bacterial plasmid has previously been described with such a high proportion of IS elements. At least four new IS elements were identified. Fifty putative proteins show no significant homology to proteins of known function; of these, eighteen have a G + C content of less than 40%, typical of known virulence genes on the plasmid. These eighteen constitute potentially unknown virulence genes. Two alleles of *shet2* toxin and five alleles of *ipaH* were also identified on the plasmid. Thus, the plasmid sequence suggests a remarkable history of IS-mediated acquisition of DNA across bacterial species. The complete sequence will permit targeted characterization of potential new *Shigella* virulence determinants.

NOVEL DOMAIN ORGANIZATION OF AngM, A NONRIBOSOMAL PEPTIDE SYNTHETASE ESSENTIAL FOR SIDEROPHORE BIOSYNTHESIS IN *VIBRIO ANGUILLARUM.*

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*Vibrio anguillarum* causes a terminal hemorrhagic septicemia in salmonid fishes. Many isolates of this bacterium possess a plasmid-mediated iron uptake system whose presence is responsible for virulence. Strains harboring the 65 kilobase pair (Kb) plasmid pJM1 are able to grow in iron-limited media containing a variety of iron chelators. However, strains cured of this plasmid could not grow under such conditions and were no longer virulent. During growth under iron-limited conditions, a system is induced in strains containing the pJM1 plasmid which results in the energy-dependent uptake of iron by the *V. anguillarum* cells. This system includes a water-soluble 348 Da siderophore, anguibactin, which accumulates in the culture medium, and a transport protein complex, whose presence is correlated with the binding and transport of iron as a complex with the siderophore anguibactin into the cell cytosol.

We have identified and sequenced a gene from the *V. anguillarum* plasmid pJM1 that encodes a 78 kDa nonribosomal peptide synthetase (NRPS) termed AngM, which is essential in the biosynthesis of anguibactin.

The mechanisms of biosynthesis by nonribosomal peptide synthetases is an RNA-independent template chain growth process with an assembly line organization of catalytic and carrier protein domains whose placement and function determine the number and the sequence of the amino acids incorporated into the peptide product. Conserved domains can be identified in peptide synthetases and each domain can act as an independent enzyme in the catalysis of a specific step in the peptide synthesis. NRPSs have been found to be involved in the biosynthesis of several secondary metabolites such as antibiotics, siderophores and toxins.

The AngM amino acid sequence shows regions of homology to the consensus sequence for the peptidyl carrier protein (PCP) and the condensation (C) domains of NRPSs. The consensus sequence for the PCP domain is DxFFxLGGDSL, where the serine is highly conserved and acts as the cofactor-binding site. The PCP domain of AngM (DDFFEMGGHSL) is assumed to be involved in tethering of activated cysteine via a phosphopantetheinyl arm bound at the conserved serine. The C domain (HHMIIDEW) is thought to be involved in the formation of the peptide bond between cysteine residue and dihydroxybenzoic acid (DHBA). The second histidine in the condensation domain has been shown to be essential for the function of the domain.

By using site-directed mutagenesis of the *angM* gene we were able to generate specific amino acid changes of the conserved residues in the PCP and C domains, which resulted in an anguibactin -deficient phenotype, demonstrating the essential role that these two domains of AngM play in siderophore biosynthesis.

The *angM* gene is located downstream of a transposase gene and they are transcribed in the same orientation. The transcriptional start site of the *angM* mRNA was determined by primer extension experiments. Furthermore, the promoter leading *angM* transcription was defined by deletion analysis of a clone containing a fragment, carrying

the transposase gene including its upstream region and the 5'end of the *angM* gene, in front of a promoterless *cat* gene.

Ribonuclease protection assays allowed us to determine that iron, the AngR protein and the TAF factor(s) regulate expression of this novel gene.

# COMPLETE NUCLEOTIDE SEQUENCE OF pMAC, A PLASMID FROM *ACINETOBACTER BAUMANNII* 19606 THAT ENCODES ORGANIC PEROXIDE RESISTANCE

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*Acinetobacter baumannii* is a piliated, gram negative opportunistic pathogen responsible for severe nosocomial and community-acquired infections. This human pathogen causes a wide range of diseases, including septicemia, endocarditis, meningitis, and respiratory, urinary, and skin wound infections. *A. baumannii* can resist a wide variety of antibiotics at very high doses. This emerging multiple antibiotic resistance phenotype has proven to be a major challenge for physicians to treat infections caused by this pathogen in compromised patients. *A. baumannii*, along with other members of this genus, is known to harbor a variety of different plasmids of various sizes. Previous work has shown that *A. baumannii* strains isolated from the same hospital outbreak can vary extensively in plasmid content, giving researchers the ability to type strains by their plasmid content. While this species is known to possess multiple plasmids in any given isolate, little work has been done to investigate the genetic structure and function of these plasmids. Given that a significant number of genes encoding resistance to antimicrobial compounds are carried on mobile genetic elements including plasmids, we undertook the task of determining the plasmid content of the *A. baumannii* 19606 prototypical strain.

Shotgun cloning and automated nucleotide sequencing of plasmid DNA isolated from *A. baumannii* 19606 revealed the presence of two different extrachromosomal elements. The plasmid reported here, pMac, is a 9,540-bp plasmid that possesses eleven predicted open reading frames (ORFs). Ten of these ORFs are transcribed in the same direction. *In silico* analysis of the ORFs revealed that at least two proteins related to plasmid replication and mobilization are encoded on pMac. The first of these is a 936-nt ORF coding for a 312 amino acid protein that shows strong similarity to replication proteins from plasmids from *Pseudomonas aeruginosa* and *Neisseria meningitidis*. A potential mobilization protein is encoded by an 1170-nt ORF coding for a 390 amino acid protein that shows high similarity to MobA from RSF1010. There are three ORFs present on pMac that code for proteins that share similarity to potential proteins encoded by plasmids from other bacteria. There is a 519-nt ORF encoding a 173 amino acid protein that is highly similar to a protein from pAM10.6 in *Pseudomonas fluorescens*, which has no assigned function. In addition, there are two ORFs in pMac that code for proteins that show strong similarity to two separate proteins from pPCP1 in *Yersinia pestis*. The first of these is a hypothetical protein of 119 amino acids that is encoded by a 357-nt ORF. The second is a predicted 101 amino acid protein that appears to be a putative transcriptional regulator. There are also three separate ORFs in pMac whose translation products possess no significant homology to any protein in Genbank. These three ORFs are 336-nt, 513-nt, and 765-nt that potentially code for proteins of 112, 171, and 255 amino acids respectively.

Interestingly, pMac possesses an 765nt ORF encoding a 255 amino acid protein that is highly similar to Ohr, which is responsible for organic peroxide resistance in *P. aeruginosa* and *Xanthomonas campestris*. This ORF is immediately upstream of a 432-nt ORF that codes for a 144 amino acid protein that is related to the MarR family of transcriptional regulators. *P. aeruginosa* and *X. campestris* share similar genetic organization by having a *marR* regulator next to *ohr*. However, in these organisms the regulator is found upstream of *ohr*. Even more striking is the fact that this appears to be the first report of *ohr* being encoded on a plasmid, since other reports show that organic peroxide resistance is mediated by chromosomal genetic elements. This finding suggests that organic peroxide resistance, a potential virulence factor, can be transferred via pMac or other *ohr*-containing plasmids.

To investigate if expression of this *ohr* homolog supplies resistance to organic peroxide generating compounds, we performed disk diffusion assays using cumene hydroperoxide (CHP) and tertiary-butyl hydroperoxide (*t*-BHP). These assays showed that *A. baumannii* 19606 was resistant to these compounds, although to lower levels than those detected in the positive control, *P. aeruginosa* PAO1. In addition, *Escherichia coli* DH5α, harboring a clone containing the *ohr/marR* locus, expressed higher resistance to CHP and *t*-BHP than DH5α cells containing the pBR325 cloning vector. Liquid cultures of *E. coli* DH5α harboring the clone containing the *ohr/marR* locus also had a higher minimum inhibitory concentration (MIC) to CHP and *t*-BHP than *E. coli* DH5α harboring pBR325. However, the levels of resistance seen in the *E. coli* clone were not as high as those seen in the *A. baumannii* 19606. This suggests that there may be another component(s) in this strain that could contribute to this resistance, such as one of the proteins encoded on pMac that has no significant homology to other known or predicted functions. Another explanation for the different levels of resistance is that the *ohr/marR* locus may not be expressed at the same levels in *E. coli* DH5α. We also determined if Ohr was specific for organic peroxides or if it would provide resistance to other peroxide generating compounds such as paraquot and $H_2O_2$. Both disk diffusion assays as well as liquid MICs demonstrated that *A. baumannii* 19606 as well as the *E. coli* DH5α clone harboring the *ohr/marR* locus were sensitive to $H_2O_2$ at levels where *P. aeruginosa* PAO1 was able to grow, indicating the *A. baumannii* 19606 Ohr is not supplying resistance to $H_2O_2$. Similar assays conducted with paraquot showed that *A. baumannii* 19606 is resistant to this compound to levels higher than that of *P. aeruginosa* PAO1. However, the *E. coli* DH5α clone harboring the *ohr/marR* locus did not supply any resistance above that seen in *E. coli* DH5α harboring the cloning vector pBR325. These results demonstrate that Ohr supplies resistance only to organic peroxide generating compounds.

Our data indicate that pMac, which seems to represent a new plasmid incompatibility group, may participate in the expression and transfer of functions that facilitate the survival of this bacterial pathogen under adverse conditions, such as those found in a human host.

VARIATION OF PLASMIDS FROM GLYCOPEPTIDE RESISTANT *Enterococcus faecium* ISOLATED FROM PIGS IN DENMARK.

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Glycopeptide resistant *Enterococcus faecium* (GRE) are a frequent cause of hospital infections. Here they can cause skin and urinary tract infections of hospitalized patients. The glycopeptide resistance is often caused by the use of the glycopeptide **vancomycin** to treat multi resistant staphylococci. However, a similar glycopeptide, **avoparcin,** was until 1995 used extensively as a growth promoter in animal production in Denmark as well as most other European countries. Resistance towards avoparcin causes simultaneously resistance to vancomycin. Hence, GRE was found in high numbers among isolates from animals in the mid-nineties. Especially among isolates from poultry (app. 72 % resistance) and slaughter pigs (app. 20 % resistance). It has previously been shown that the GRE isolates from pigs in Denmark originate from the same clone, as evaluated by Pulsed Field Gel Electrophoresis (PFGE). This clone was resistant towards erythromycin and tetracycline in addition to vancomycin.

For a more thorough analysis of the clone, we picked 5 of the GRE isolated from slaughter pigs from each of the years 1997, 1998 and 1999. The genes encoding vancomycin (*vanA*) and erythromycin *erm*(B) resistance was located on the same plasmid of app. 175,000 base pairs, present in all our porcine isolates. These plasmids were digested with restriction endonucleases and subsequently studied by Restriction Fragment Length Polymorphism (RFLP). The analysis clearly showed, that the plasmids are not all identical, even though they originate from the same bacterial background, thus have identical (chromosomal) PFGE type. The plasmids seem to belong to several different sub-groups, with distinctive RFLP patterns. Therefore, it is possible to use this method to separate the different isolates, even though they have the same PFGE pattern. This could potentially be used to trace the spread of the clone within pig populations and to trace the spread to *E. faecium* in other reservoirs, such as humans.

# CHARACTERIZATION OF A LARGE PLASMID, ENCODING STREPTOGRAMIN RESISTANT, FROM AN *ENTEROCOCCUS FAECIUM* OF ANIMAL ORIGIN

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The streptogramin antibiotic virginiamycin was until 1998 used as a growth promoter in the food animal production in Europe and is still used in the United States as growth promoter. Recently, another streptogramin (quinopristin/dalfopristin) was released for treatment of infections in humans with methicillin resistant *Staphylococcus aureus* and glycopeptide resistant *Enterococcus faecium*. Among enterococci, *Enterococcus faecalis* is intrinsically resistant to streptogramins while *Enterococcus faecium* becomes resistant by acquisition of horizontally transferable resistance genes. Streptogramins consist of two chemically distinct compounds; streptogramin A and streptogramin B. Streptogramin A resistance is encoded in enterococci by the *vat*(D), *vat*(E) or *vgb*(A) genes while streptogramin B resistance is encoded by the macrolide-lincosamide-streptogramin B ($MLS_B$) resistant gene *erm*(B).
In an *E. faecium* isolate (F9631160-1) of poultry origin a large plasmid (>150 kb) was identified. On this plasmid the *vat*(D) and the *erm*(B) gene was positioned with a distance of 2,0 kb. Resistance to streptogramin could be transferred to a well-characterized plasmid free *E. faecium* (BM4105) both by horizontal transfer of the plasmid and presumable by transposition of a mobile DNA element containing both resistance genes. Variations in size of positive fragments on Southern blots of *Eco*RI digested total DNA of donor and transconjugants using both resistance genes as probes revealed transposition. The physical link between the *erm*(B) and *vat*(D) genes was unaffected by the transfer of resistance to the recipient. A 6421 bp *Eco* RI fragment of the original donor plasmid containing both resistance genes was sequenced and the obtained sequence was compared to sequences obtained from two transconjugants to characterize the presumed transposon-like element residing on the original plasmid. Results are presented here.

# ORGANIZATION OF THE PLASMID *CPE* LOCUS IN *CLOSTRIDIUM PERFRINGENS* TYPE A STRAINS

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*Clostridium perfringens* type A isolates causing food poisoning have a chromosomal enterotoxin gene (*cpe*), while *C. perfringens* type A isolates responsible for nonfoodborne human GI diseases carry a plasmid *cpe* gene. In the current study, the plasmid *cpe* locus of type A, nonfoodborne disease isolate F4969 was sequenced to design primers and probes for comparative PCR and Southern blot studies of the *cpe* locus in other type A isolates. Those analyses determined that the region upstream of the plasmid *cpe* gene is highly conserved among type A isolates carrying a *cpe* plasmid. Organization of the type A, plasmid *cpe* locus was also found to be unique, as it contains IS*1469* sequences located similarly to those in the chromosomal *cpe* locus but lacks the IS*1470* sequences found upstream of IS*1469* in the chromosomal *cpe* locus. Instead of upstream IS*1470* sequences, a partial ORF potentially encoding cytosine methylase (*dcm*) was identified upstream of IS*1469* in the plasmid *cpe* locus of all tested type A isolates. Similar *dcm* sequences were also detected in several *cpe*-negative *C. perfringens* isolates carrying plasmids, but not in type A isolates carrying a chromosomal *cpe* gene. Contrary to previous reports, sequences homologous to IS*1470,* rather than IS*1151*, were found downstream of the plasmid *cpe* gene in most tested type A isolates. Those IS*1470*-like sequences reside in about the same position, but are oppositely-oriented and defective, relative to the IS*1470* sequences found downstream of the chromosomal *cpe* gene. Collectively, these and previous results suggest the *cpe* plasmid of many type A isolates originated from integration of a *cpe*-containing genetic element near the *dcm* sequences of a *C. perfringens* plasmid. The similarity of the plasmid *cpe* locus in many type A isolates is consistent with horizontal transfer of a common *cpe* plasmid among *C. perfringens* type A strains.

# MOLECULAR CHARACTERIZACION OF PLASMIDS ENCODING EXTENDED-SPECTRUM ß-LACTAMASES (ESBLs) FROM NOSOCOMIAL ENTEROBACTERIAL ISOLATES.

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Although there is a variety of mechanisms of bacterial resistance to beta-lactam antibiotics, the most important one is the production of beta-lactamases inactivating penicillins and cephalosporins. The classification of beta-lactamases is based on biochemical, enzymological (i.e. molecular structure, inhibitory property, substrate-profile, relative rate of hydrolysis) and immunological characteristics. Extended-spectrum beta-lactamases (ESBLs) can be derived from plasmid TEM or SHV enzymes. These enzymes have now been sequenced and it has been found that relatively few point mutations have occurred in the gene of the TEM (pI 5.4 - 5.9) and SHV (pI 7.0 – 8.2) type enzymes. The amino acid mutations can alter the conformation, the active site and change the hydrance of beta-lactamase-cephalosporin binding capacity. So the enzyme is able to bind and hydrolyze the third generation cephalosporins. Successive mutations interact radically increasing the binding capacity of enzymes and confer resistance to newer cephalosporins. The use of these drugs provides a strong selective pressure to develop these mutations. There is also a small but growing family of plasmid-mediated ESBLs that are not related to TEM or SHV ß-lactamases, such as TLA-1 with a pI of 9.0. Sporadic nosocomial outbreaks due to strains producing an ESBL are one of the main epidemic problems in some hospitals resulting in a concurrent dissemination of genes, plasmids or strains. In this work we characterized the extended-spectrum ß-lactamases encoded in multiresistant clinical isolates of enterobacteria in Mexico.

One hundred and thirty-five non repetitive clinical isolates of enterobacteria ESBL producers, were collected from 9 different hospitals during 8 years in Mexico. Two major groups of ß-lactamase profiles were detected: Group A containing 119 strains that expressed enzymes with pI of 5.4, 7.6 or 5.4, 8.2; and group B conformed by 16 clinical isolates expressing pI of 5.4, 9.0. In all patterns the last enzyme corresponded to an ESBL with the ability to hydrolyze cefotaxime. From these two groups 29 isolates were selected to conform this study. Group $A_1$ with 13 of 119 strains and group B with 16 strains. PCR assays with three different sets of oligonucleotides were used to amplify a partial region of the *tem* gene and the *tla* and *shv* complete genes. These two last products were used for nucleotide sequencing. PFGE was performed using *Xba*I to determine clonally relatedness and bacterial conjugations in order to determine transferability of the resistance to cefotaxime.

From group A, 5/13 (38%) strains coded for the *shv-2* gene: *K. pneumoniae* (4), and *E. coli* (1); the *shv*-5 gene was detected in 8/13 (61.5%): *K. pneumoniae* (6), *E. coli* (1), and *E. cloacae* (1). Concerning group B, the *tla* gene was identified in 16/16 (100%) strains: *Escherichia coli* (9), *Enterobacter cloacae* (3), *Klebsiella pneumoniae* (2),

*Enterobacter* spp (1) and *Citrobacter freundii* (1). The presence of TEM ß-lactamase (pI of 5.4) was associated with SHV-derived in 93% and TLA-1 in 62% enzyme profiles. PFGE showed different electrophoretic patterns among different enterobacteria groups. All clinical isolates harbored more than one plasmid, 18 of the 29 isolates studied were able to transfer the resistance to cefotaxime by conjugation with the largest plasmid observed (>100 kb).

When plasmids expressing ESBLs from group A and B were digested with *Dra*I or *Pst*I, a common restriction pattern was identified in each respective group. In order to detect a possible relation among the restriction pattern and the ESBL genes, a Southern hybridization was performed with specific probes of *shv* and *tla*. Results showed the same banding pattern in all plasmids coding for each ESBL group.

These results suggest the spread of a high conserved region of DNA encoding the ESBL in all these plasmids which were obtained from non-related clinical isolates collected from 9 different hospitals during an eight year period. It is of our interest to characterize this fragment of DNA in order to understand the evolution of ESBLs in multi-resistant clinical isolates of enterobacteria.

# *PATHOGENICITY ISLANDS/GENE TRANSFER ELEMENTS*

## ORAL PRESENTATIONS

**172** Mobile genetic elements encoding toxinosis-causing bacterial toxins: the superantigen-encoding pathogenicity islands of *S. aureus*. Richard P. Novick

**173** Comparative bacteriophage genomics. Graham F. Hatfull, Mike Ford, Jen Houtz, Marisa Pedulla and Roger Hendrix

**174** Constraints on lateral gene transfer in prokaryotes. Jeffrey Lawrence

**175** Environmental and genetic control of the SXT, a conjugative antibiotic resistance gene transfer element from *Vibrio cholerae*. John W. Beaber, Bianca Hochhut and Matthew K. Waldor

## POSTER PRESENTATION

**176** Identification and analysis of transposable genetic elements of *Paracoccus* spp. Dariusz Bartosik, Marta Sochacka, Michal Szymanik, Jacek Lukasik, Jadwiga Baj and Miroslawa Wlodarczyk

MOBILE GENETIC ELEMENTS ENCODING TOXINOSIS-CAUSING BACTERIAL TOXINS: THE SUPERANTIGEN-ENCODING PATHOGENICITY ISLANDS OF *S. AUREUS*. Richard P. Novick, Skirball Institute, NYU School of Medicine, New York, NY, 10016, USA.

It is a remarkable observation that virtually all bacterial toxins associated with specific clinical conditions (toxinoses) are encoded by mobile (and therefore variable) genetic elements. Remarkably, these rarely, if ever, carry determinants of antibiotic resistance. Examples are the toxins responsible for diphtheria, anthrax, tetanus, botulism, cholera, toxic shock, scarlet fever, exfoliative dermatitis, food poisoning, travelers' diarrhea, shigella dysentery, necrotizing pneumonia, and others. A recently discovered example of this phenomenon is the family of related staphylococcal pathogenicity islands encoding superantigens (SAgs). These are 15-20 kb elements that occupy constant positions in the chromosomes of toxigenic strains, and are characterized by certain phage-related features, namely genes encoding integrases, helicases, and terminases, and the presence of flanking direct repeats. The prototype, SaPI1 of *S. aureus*, encodes TSST-1 plus two newly described SAgs, SEK and SEL. Other members of the family encode enterotoxins B (SaPI3) and C (SaPI4), plus at least two other SAgs each. SaPI1 and SaPI2, also encoding TSST-1, are excised and induced to replicate by certain staphylococcal phages, and are then encapsidated at high efficiency into phage-like infectious particles with heads about 1/3 the size of the helper phage heads, commensurate with the sizes of the respective genomes. This results in transfer frequencies of the order of $10^8$ /ml, and is presumably responsible for the spread of these elements as well as for their acquisition in the first place. In the absence of a helper phage, these islands are highly stable; neither excision, loss, or transfer occurs at detectable frequency. Several general implications of this phenomenon will be discussed. One is that the determinants of these toxins have been imported from other species and therefore are not components of the basic genome of the extant producing organisms. This raises the question of the biological (adaptive?) roles of these toxins. Another is that the toxin-carrying units can spread among different (though probably related) species. An interesting question is that of the biological basis for the separation of toxin and resistance determinants.

COMPARATIVE BACTERIOPHAGE GENOMICS

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It is estimated that the majority of life forms in the biosphere are bacteriophages with a total number of approximately $10^{31}$ particles. The extent of the diversity of this population is not clear, although it is becoming apparent that it is probably quite substantial. To examine the extent of this diversity, the manner in which bacteriophage genomes are related to each other, and how they have evolved, we are conducting a comparative genome analysis of several groups of bacteriophages.

One group of phages that we are studying is the mycobacteriophages, viruses that infect fast-growing non-pathogenic species such as *Mycobacterium smegmatis* or slow-growing strains such as *Mycobacterium tuberculosis* or the avirulent vaccine strain BCG. We have sequenced the genomes of three previously described phages (L5, D29, TM4) and 11 newly isolated phages from a variety of geographic locations (Barnyard, Bxb1, Bxz1, Bxz2, Cjw1, Corndog, Che8, Che9c, Che9d, Omega, Rosebush). These genomes suggest a large degree of diversity among the mycobacteriophages, and contain a large number of genes that are not present in other phages, or elsewhere in sequence databases.

A second group of phage genomes that we have characterized are those infecting gram-negative bacterial species and include HK022, HK97, N15, N4, Mu, phiK02, ES18 and P22. These phages, along with the mycobacteriophages, reveal what is perhaps the most characteristic feature of their genomic architectures: that they are highly mosaic, and appear to be constructed of modules consisting of genes, groups of genes or gene domains, each of which may appear in different phage genomes and assembled in different combinations. Any explanation as to how these viruses have evolved must include an accounting of this pervasive mosaicism. We suggest that lateral DNA transfer events between infecting phages and resident prophages represent a dominant part of this process.

Given the apparent fluidity in this dynamic population of viral genomes it should perhaps not be surprising that the phage genomes that we have sequenced carry a significant number of genes that were not previously associated with phage genomes. Moreover, it is apparent that there is no clear distinction between phage genomes and plasmid genomes, since both may share a large number of common genes. In an extreme example, the genome of bacteriophage N15 has one-half that contains genes required for the viral structural and assembly and another half that contains genes involved in the replication of an extrachromosomal linear prophage. We find many additional examples of viral genomes that carry genes previously associated with plasmid molecules and it is clear that phages and plasmids participate freely in genetic exchange.

# CONTSRAINTS ON LATERAL GENE TRANSFER IN PROKARYOTES

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Unlike crown eukaryotic species, microbial lineages are created by continual processes of gene loss and acquisition promoted by horizontal genetic transfer. The amount of foreign DNA in bacterial genomes, and the rate at which it is acquired, is consistent with gene transfer being the primary catalyst for microbial lineage differentiation. However, when measured by several independent methods, the rate of successful gene transfer appears to vary among bacterial lineages. Genetic headroom reflects the amount of potentially dispensable information – reflected in codon usage bias and codon context bias – which bears low selective coefficients; this information can be readily superceded by functions introduced by gene transfer. Novel methods have been developed for quantitating genetics headroom (codon usage and context biases) that allows for comparison across genomes. We have found that the heterogeneity in foreign DNA content across microbial lineages is directly correlated with amount of genetic headroom intrinsic to a bacterial genome. Therefore, not all bacterial lineages offer the same capacity to serve as successful recipients of horizontally transferred DNA. Since the information encompassed by genetic headroom can be transiently sacrificed to allow experimentation with acquired functions, this process may facilitate the exploration of new ecological roles. In this way, genetic headroom offers potential metric for the propensity of a lineage to speciate.

In addition, the likelihood of successful horizontal transfer may be a function of phylogenetic distance, whereby more closely related taxa have a higher probability of exchanging genes - even by illegitimate processes that are independent of the degree of nucleotide sequence divergence - than do more distantly related lineages. That is, different lineages may not be equivalent in their ability to act as potential donors for horizontal gene transfer, even if the genes they bear offer the same physiological benefit. This gradient in the potential to act as a DNA donor may be due to the presence of strand-specific, asymmetrically-distributed sequences which control DNA replication by slowing the replication apparatus in the vicinity of the replication terminus. Such sequences appear to be shared among more closely related lineages, but comprise non-overlapping sets among distantly related sequences. As a result, incoming DNA from distantly-related taxa may bear sequences which would interfere with DNA replication in their new host genome, thereby reducing the probability of a successful transfer. Both mechanisms serve to shape the flow of genes among bacteria by lateral transfer processes.

# ENVIRONMENTAL AND GENETIC CONTROL OF THE SXT, A CONJUGATIVE ANTIBIOTIC RESISTANCE GENE TRANSFER ELEMENT FROM *VIBRIO CHOLERAE*

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Conjugative DNA transfer facilitates lateral spread of antibiotic resistance genes in bacterial populations. We have sequenced the DNA of SXT, a conjugative chromosomally integrating antibiotic resistance gene transfer element that has recently become widespread in Asian *Vibrio cholerae*. This ~100 kb element appears to be a chimera composed of transposon-associated antibiotic resistance genes linked to a variety of plasmid and phage-related genes. Functional studies revealed that the element's plasmid-related genes are required for SXT conjugation and its phage-related genes are required for regulation of its transfer, chromosomal integration and excision. We have demonstrated that induction of the bacterial SOS response significantly increases the frequency of transfer of SXT in a manner analogous to induction of the lytic cycle in phage lambda. Activation of the SOS response results in diminished repression by the SXT repressor, SetR, on the conjugation transcriptional activators, *setC* and *setD*. These results suggest that treatment of bacteria harboring SXT and related elements with antimicrobial agents that induce SOS could promote the dissemination of resistance genes.

# IDENTIFICATION AND ANALYSIS OF TRANSPOSABLE GENETIC ELEMENTS OF *Paracoccus* spp.

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Insertion sequences (IS), which are the simplest form of mobile genetic elements, are the most recombinogenic factor of bacterial genomes. Transposition of these elements usually involves structural changes in DNA that lead to the formation of insertion mutations, deletions, inversions and translocation (of even large fragments of DNA), which may result in varied phenotypic effects (1). The results of sequence analysis of several bacterial genomes suggest that insertion sequences are more widely spread on plasmids than on chromosomes. Plasmids thus seem to be a reservoir of transposable elements, propagating them by horizontal transfer between various bacterial populations. These observations encouraged us to undertake complex studies aimed at the identification, localization (plasmid/chromosome) and distribution of IS in bacteria of the genus *Paracoccus* (α Proteobacteria), which currently embraces fourteen species. Prior to beginning the study the only known insertion sequence of *Paracoccus* spp. was IS*1248* of *P. denitrificans* PdX13, which is classified to the family IS*5* (5). In the first stage of our studies we constructed a novel shuttle entrapment vector pMEC1, which enables direct *in vivo* capture of transposable mobile elements in paracocci. To construct the vector we chose the small cryptic plasmid pWKS1 (2.7 kb) of *Paracoccus pantotrophus* DSM 11072, which is stably maintained in all paracocci and is compatible with natural plasmids of these bacteria. The entrapment vector pMEC1, is composed of (a) the replicator region of pWKS1, (b) *E. coli*-specific mobilizable vector pABW1 ($Km^r$; *oriT* RK2; [2]) and (c) entrapment cartridge (obtained from plasmid pGBG1; [4]). This cartridge carries a silent tetracycline resistance gene under control of *pR* promoter of bacteriophage λ, and gene coding for λ CI repressor. Inactivation of repressor, or operator (e.g. through insertion of a mobile genetic element), results in constitutive expression of the tetracycline resistance. The functionality of pMEC1 was tested in different strains of *P. pantotrophus* (DSM 11072, DSM 11073, DSM 65, LMD 82.5), *P. methylutens* DM12, *P. solventivorans* (DSM 11592, DSM 6637) and *P. versutus* UW1. Both kanamycin and tetracycline markers (present in pMEC1) are suitable for selection in the above species. $Tc^r$ clones appeared with frequency from $10^{-7}$ to $10^{-3}$, depending on the strain. Basing on the sequence of the selective cartridge (*cI* gene) we designed 5 pairs of primers, whose use in PCR reactions enabled (a) precise localization of IS in the cassette, (b) determination of accurate size of IS and (c) choice of appropriate starters for sequencing. We have begun a detailed analysis of chosen insertion elements. So far we have sequenced only a few of them. We found that the tested paracocci contain several already known elements, for instance IS*1248* of *Paracoccus denitrificans* (Acc. no. U08856) is present in *P. methylutens* DM12, *P. solventivorans* DSM 11592 and *P. pantotrophus* DSM 11072; IS*1247* of *Xanthobacter autotrophicus* (Acc. no. X84038) was found in *P. methylutens* DM12; Tn*5393* (Acc. no. AF262622) was identified in *P.*

*pantotrophus* LMD 82.5. Several novel IS have been also identified, e.g. IS*Pp1* (1.4 kb; IS*256* family) and IS*Pp2* (1 kb; IS*5* family) of *P. pantotrophus* DSM 11072; IS*Ps1* (1.5 kb; IS*256* family) of *P. solventivorans* DSM 11592. Analysis of the nucleotide sequences of the above-mentioned ISs allowed determining their structure, that is to identify the transposase gene, terminal inverted repeats as well as target sequence. An analysis was undertaken to determine the copy number and localization of the individual sequences in the host genome, as well as their dissemination in other *Paracoccus* species.
Analysis of the available sequences of paracoccal plasmids revealed the presence of two additional novel IS elements. One of them, IS*Pv1* (similar to ISXCD1 from *Xanthomonas campestris*; Acc. no. AF263433), is present in the composite plasmid pTAV1 of *P. versutus* UW1. The second one is IS*Pm1* (0.8 kb; IS*5* family) of pMTH3 (approx. 23 kb) of *P. methylutens* DM12. The analysis of plasmid pMTH3 done by us suggests that IS-mediated structural rearrangements occurred in this plasmid. We constructed a mini-replicon of the plasmid (pMTH100; 5.3 kb), containing two functional regions – responsible for replication of the plasmid (REP; with similarity to the replicon of pSW500 of *Pantoea stewartii* [3]) and its stable maintenance (STA; with a novel addiction system) as well as the mentioned above insertion sequence IS*Pm1*. IS*Pm1* is located in the terminal part of pMTH100, downstream of gene *rep* (coding for replication initiator), what suggests possibility of co-transcription of genes responsible for replication and transposition. The structure of plasmid pMTH3 seems to be unusual since a considerable part of its genome is in the form of two long repeated sequences (head to head oriented). The terminal parts of mini-replicon pMTH100 were shown to contain approx. 200 bp-long identical inverted repeats (one of which contains a duplicated terminus of IS*Pm1*), which are the beginning of the mentioned above repeated sequences of pMTH3. We also present the results of our studies that were undertaken to verify the hypothesis that the insertion of IS*Pm1* into pMTH3, followed by intra-replicon structural rearrangements, resulted in the formation of a natural transposon, containing REP and STA modules of the analyzed plasmid.

We believe that the started analysis will provide a lot of information on the frequency and direction of horizontal gene transfer in this group of bacteria and will also allow to conclude on the role of plasmids in this process. The obtained results may also be of use in further general considerations relating to the evolution of bacterial genomes.

ACKNOWLEDGEMENTS
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# *PLASMID AS TOOLS*

## ORAL PRESENTATIONS

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# ANTISENSE RNA BASED BACTERIOPHAGE DEFENSE STRATEGIES

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The dairy industry has extensively utilized strains of *Streptococcus thermophilus* as starter cultures or culture adjuncts for the manufacture of a variety of fermented dairy products, including yogurt and specialty cheeses. Lytic phages infecting these cultures are subject to both vertical and horizontal evolution. The vertical evolutionary component is the gradual accumulation of spontaneous mutations, predominantly point mutations but also short insertions and deletions, that are passed from parent to progeny. Horizontal evolution is primarily the result of recombination between interbreeding phage populations, including resident prophages and remnants thereof, but may also occur between an invading phage and the host genome. As a result, two parent phages produce a chimeric third. These events, compounded with the influx of new phages present in pasteurized milk, enable the emergence of subpopulations of phages capable of subverting previously resistant starter cultures. Depending upon the severity and temporal progression of the infection, the concomitant loss of fermentative capacity associated with culture lysis can significantly retard or halt batch fermentations; thereby inflicting significant losses of time and production capital each year. These losses are particularly severe when highly specialized strains, which are themselves the valuable product of scientific discovery and product development, become susceptible to phage attack. In this case, the costs committed for strain development will not be recovered if the expected lifetime of a new, highly specialized strain is diminished by the appearance of virulent phages capable of attacking it. As traditional methods for the isolation of phage insensitive mutants continue to fail to produce *S. thermophilus* strains with long term utility, the dairy industry has turned to genetic engineering to expedite the development of novel and more efficacious phage defense strategies. The expression of antisense RNA targeted against phage-encoded genes has recently emerged as a strategy for the construction of phage resistant strains.

Mechanistically, antisense RNA binds to the complementary, phage-encoded sense mRNA strand and creates a translationally inactive double stranded RNA (dsRNA) molecule. Formation of the dsRNA molecule is believed to silence gene expression through the cooperative action of one or more intermolecular mechanisms. If the antisense RNA includes sequences complementary to the ribosome binding site (RBS), then the formation of dsRNA may mask the RBS, preventing efficient ribosome loading and reducing translation of the targeted gene of interest. Formation of dsRNA downstream of the RBS may also interfere with translation by sterically impeding, to some degree, the procession of the mRNA through the ribosome. In addition, the formation of dsRNA may destabilize the sense mRNA by promoting the action of dsRNA-specific ribonucleases. Lastly, if the gene of interest is transcribed on a

polycistronic mRNA, then antisense targeting may also negatively impact the expression of genes located up- or downstream of the target gene, thereby causing deleterious pleotropic effects that might further inhibit phage proliferation.

In this study, comparative bioinformatic analyses of the whole genome sequences of six *S. thermophilus* phages revealed that genes associated with the putative Sfi21-type DNA replication module are the most suitable targets for antisense RNA since they are highly conserved at the nucleotide level (greater than 90%) and found in five of the six sequenced phages. Two of the identified genes (*orf33* and *orf38*) were chosen for further study. Antisense RNA cassettes of varied length (ranging from 503- to 1,512-bp) were constructed from various structural and putative regulatory regions of *orf33* and *orf38*. These cassettes were expressed from pTRK687, a pNZ123-based, high-copy-number RNA expression vector, and tested for their relative ability to impede the proliferation of six previously uncharacterized phages. The results of these studies illustrated that antisense targeting of highly conserved, early-expressed genes can be utilized as efficacious phage defense with widespread utility.

# THE COPY-CONTROL EXPRESSION VECTORS (pBAC/oriV/trfA) WITH THE TIGHTLY CONTROLLED *ara* AND *rha* PROMOTERS

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To attain high level of protein synthesis, most of the available expression vectors employ high-copy(HC) plasmids carrying a strong and inducible promoter. To their disadvantage, however, such HC vectors usually exhibit a high background expression at the non-induced state. Since background expression (which often is toxic to the host-vector system) should be proportional to the copy number, we decided to construct a new kind of copy-control expression vectors by re-engineering our conditional single-copy(SC) pBAC/oriV vectors [see Wild, J., Hradecna, Z. and Szybalski, W.: Single-copy/high-copy(SC/HC) pBAC/oriV novel vectors for genomics and gene expression. Plasmid 45 (2001)142-143]. The pBAC/oriV vectors allow "on command" amplification of vector and cloned DNA. For amplification, which is a switch from SC to HC number, we employed the *oriV*/TrfA replication function of the broad-host-range plasmid RK2. We adopted this novel switch for construction of a set of copy-control expression vectors with dual replicons. The SC state of copy-control expression vectors is based on the F'-derived replicon of BAC vectors, whereas the HC state can be achieved at wish by switching to the *oriV*/TrfA amplification function of RK2.

To permit the use of various hosts, we placed all the control and replication elements on one plasmid. These included both *cis* and *trans* elements that provided **(i)** very stable SC maintenance, **(ii)** inducible and efficient switch to the HC state, and **(iii)** very tightly controlled and inducible promoters for the high-level gene expression.

**(i)** The SC replication and maintenance elements included the *oriS* and *repE* of the F' plasmid together with the *parABC* partition determinants. The SC mode of maintenance assures high stability of our uninduced pBAC/oriV/trfA vectors, because of the nearly undetectable expression background; this is especially important when the leaking product is toxic to the host. It is well known that it is often not possible to clone some "toxic" genes into the HC plasmids, or stably maintain them after cloning. Such HC clones, even when the cloned gene is in the uninduced (repressed) state, do undergo progressive "evolution" by acquiring mutations or other undesirable spontaneous modification when the cloned gene expresses a sufficiently high background levels of a toxic product.

**(ii)** The HC replication originating at *oriV* was based on the HC mutants of gene *trfA* that was placed under control of an inducible promoter. Only upon addition of the inducer, the newly synthesized TrfA was initiating replication at *oriV*.

**(iii)** For gene expression we used the *araC-*$P_{BAD}$ promoter or other tightly controlled promoters, including the *rhaS-*$P_{rha}$ promoter. Downstream of a promoter we placed the multiple cloning site (MCS), into which we cloned either the gene to be expressed or the reporter gene, namely the entire *lacZ* gene.

We have constructed the following types of plasmids that were transformed into the *E. coli* DH10B host.

**(1)** Plasmid pJW544 carries the *araC-*$P_{BAD}$*-trfA*203 cassette with MCS downstream of the *trfA* gene. In this plasmid, the *araC-*$P_{BAD}$ promoter, when induced

with arabinose, served to express both the *trfA* gene and the *lacZ* reporter gene that have been cloned into MCS (resulting in plasmid pJW520). The uninduced levels of the *lacZ* product (βGal) were very low (2-8 Miller units). Upon induction with 0.01 % L-arabinose, copy number increased by around fifty-fold and the βGal synthesis reached 50 000 to 60 000 units.

**(2)** Whereas in plasmids pJW544 and pJW520 both the amplification and expression functions were jointly induced, plasmids described below allow these two functions to be induced independently.

(a) Plasmids pJW565 and pJW566 contained the *rhaS-*$P_{rha}$-MCS(*lacZ*) gene-expression operon (in two orientations) and the *araC-*$P_{BAD}$*-trfA*203 amplification cassette. Addition of L-rhamnose (0.02 %) was inducing the *lacZ* expression, but only from the single gene copy. Addition of L-arabinose alone (0.01 %) resulted only in the pJW565 or pJW566 plasmid amplification and permitted to compare the βGal 'leakage' for SC and HC plasmid states. The maximum expression, was obtained only when both sugars, L-arabinose and L-rhamnose, had been added.

(b) Plasmids pJW567 and pJW568 contained the *rhaS-*$P_{rha}$*-trfA*203 amplification cassette (in two orientations) and the *araC-*$P_{BAD}$-MCS(*lacZ*) gene-expression operon. Addition of L-arabinose (0.01 %) led to *lacZ* expression but only from the single gene copy. Addition of L-rhamnose alone (0.02 %) resulted only in amplification of plasmids pJW567 or pJW568, and permitted to compare the βGal leakage for SC and HC plasmid states. The maximum expression, like in case (a) above, was obtained only when both sugars, L-arabinose and L-rhamnose, had been added.

Activities of either promoter can be modulated by glucose or fucose, when added together with inducers, enhancing the versatility of the SC/HC copy-control expression systems. Adjusting concentrations of inducer and glucose or fucose allows various intermediate level of the cloned gene expression.

The major advantage of our copy-control expression vectors is their extreme tightness, as any leakage from a single gene copy (SC) is obviously fifty times lower than from the 50-copy (HC) vector, whereas upon induction, the expression is as high as from the HC expression vectors. Thus, we obtained the induction ratios reaching about five orders of magnitude.

The additional advantage of our copy-control expression vectors, as compared with various currently available HC vectors, is the enhanced viability of the host-vector system after the properly chosen induction procedure.

THE ROLE OF IHF IN P1 PLASMID REPLICATION

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The DnaA protein is required for the initiation of DNA replication from the origin of the *E. coli* chromosome, as well as from many plasmids. This requires binding of the protein to specific sites within the origin called DnaA boxes. The P1 plasmid origin contains DnaA boxes on the right and left sides (bp 386-405, 610-646 respectively), and efficient replication is possible with either set of DnaA boxes (1,2). However, we observed that plasmids containing only the right DnaA boxes require downstream sequences (bp 646-1000) to replicate that include an IHF binding site located at bases 667-679. Indeed, these plasmids are not replicated in *ihfA* (IHF) mutants.

In origins with the left DnaA boxes deleted, insertions of a few base pairs between the right DnaA boxes and the IHF site eliminated origin function. However, insertion of one full DNA turn restored origin function, indicating that the IHF site needs to be properly phased with respect to the DnaA boxes. Deletion of DNA sequences downstream of the IHF site also resulted in nonfunctional origins. Replacement of small sections of this downstream region defined the minimum sequences required for function. The possibility of protein binding to this region is being investigated by DNA footprinting since IHF is known to effect interactions between proteins at a distance by binding and bending the intervening DNA (3,4).

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**Delphi Genetics, a company developing poison-antidotes technologies**

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Delphi Genetics is Spin-off company of the Université Libre de Bruxelles (ULB) founded in November 2001.
Delphi Genetics develops technologies based on the properties of plasmidic poison antidote genes. Delphi Genetics manages a patent portfolio that includes the *ccdB* positive selection of recombinants and other applications of plasmidic poisons and antidotes.
The different applications of poisons/antidotes loci developed by Delphi Genetics will be presented.

# CHARACTERIZATION OF THE CRYPTIC PLASMID pCC1 FROM *CORYNEBACTERIUM CALLUNAE* AND ITS USE FOR VECTOR CONSTRUCTION

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Discovery of small indigenous cryptic plasmids in corynebacteria has been a prerequisite for gene manipulations in these bacteria, used for industrial production of various amino acids. Most vectors for corynebacteria are based on the related plasmids pSR1 and pGA1 from *Corynebacterium glutamicum* and on pBL1 from *C. glutamicum* ssp. *lactofermentum*. To broaden the possibilities to form biplasmid systems for amino-acid-producing corynebacteria, we searched for further plasmids compatible with the vectors based on the above mentioned plasmids. Therefore, detailed analysis of cryptic plasmid pCC1 from *C. callunae*, originally described by Sandoval et al. (1), was performed.

The complete nucleotide sequence of the plasmid pCC1 (4109 bp) was determined (GenBank Accession Number 15426257). DNA sequence analysis revealed five open reading frames longer than 200 bp. Only one of the deduced polypeptides showed significant homology with the known bacterial gene products, namely the Rep proteins encoded by some plasmids of the pC194 family of plasmids replicating by the rolling circle (RC) mechanism. Within this plasmid family, the Rep protein of pCC1 showed the highest degree of similarity to the Rep protein of another corynebacterial plasmid pBL1. These data suggest that also the plasmid pCC1 replicates by the RC mechanism.

To construct an *Escherichia coli-C. glutamicum* shuttle cloning vector based on pCC1, the 2.1-kb fragment of pCC1, carrying only its *rep* gene, was joined with the *E. coli* cloning vector pS19. The vector pS19 was constructed from the vector pK19 (2) by replacing the kanamycin resistance determinant for the streptomycin/spectinomycin resistance determinant coming from the *C. glutamicum* plasmid pCG4 (3). The resulting plasmid pSCCD1 was found to be stably maintained in population of *C. glutamicum* cells grown in the absence of selection pressure. Plasmid pSCCD1 is compatible with plasmid vectors based on plasmids pBL1 and pSR1. On the other hand, incompatibility between plasmids pCC1 and pGA1 was observed. (Plasmid pKG48, a derivative of pGA1, was lost from *C. glutamicum* cells harboring plasmid pSCCD1). Plasmid pSCCD1 is being used for cloning *C. glutamicum* genes involved in biosynthesis of amino acids.

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# RAPPORTEURS

**188** Let's sequence the floating genome! Anne Summers

**189** Complete Genome Resources at NCBI. Tatiana Tatusova

**Let's Sequence the Floating Genome!**

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It is high time to begin deliberately sequencing plasmids, bacteriophages, and other sub-chromosome-size mobile replicons including conjugative transposons rather than just having them sequenced "by accident" in the context of the genome sequencing of a single representative host bacterium. It is also essential to establish databasing (at least) and other bioinformatics tools suitable for archiving and analyzing such highly mobile and rapidly evolving elements.

Having information on these independent and mobile replicons is essential for such important enterprises as: (1) understanding the mechanisms, extent, and evolution of horizontal gene exchange - one of the biggest "new ideas" (not to those of us who work on plasmids!) to hit biology in recent years. It's even essential to understanding the evolution of prokaryotes (period!) and very likely that of some lower eukaryotes; (2) control of the spread of infectious diseases and antibiotic resistance; and (3) full exploitation of the novel metabolic and DNA manipulation capacity of prokaryotes for industrial and bioremediation applications, to name but a few areas.

Phages can be sequenced relatively easily, but sequencing plasmids is hard because they need to be separated physically from the cell chromosome before library construction. Construction of individual discrete plasmid libraries is preferable because the wide occurrence of insertion sequences make computer formation of contigs from shotgun sequencing of all the small supercoils in a CsCl gradient a risky proposition. So there is a need for new, ideally high throughput methods for preparation of pure plasmid DNA. Shouldn't be rocket science; someone just needs to do it. Other challenges have to do with the perception of low info return for labor involved in library construction, but since this latter is now roboticized that may be less an impediment.

Obviously organisms get sequenced because of pressure from "organism advocates". Plasmids and phages will only be sequenced if we come up with a coherent and focused plan for beginning the enterprise. Once the information begins to come in and be analyzed, there will be no argument but that it provides very important and commercially useful information.

Complete Genome Resources at NCBI

Tatiana Tatusova

National Center for Biotechnology Information, National Library of Medicine
National Institutes of Health, Bldg. 38A 8600 Rockville Pike, Bethesda, MD 20894

Genomes section of Entrez provides access to over 1,000 records that represent more than 900 different species including over 800 viruses, > 80 micorbial genomes, specially curated set of organelles and a number of viroids, and broad host range plasmids. The National Center for Biotechnology Information (NCBI) is providing useful access to these data through Entrez search and retrieval engine and visualize the sequence information at varying levels of detail. Primary resources include phylogenetically organized displays, precomputed relationships and direct access to analytical tools allowing evolutionary, structural and similarity-based perspectives of the data.

Examining the differences between protein sequences of various organisms gives insight into the origin of genes and the relationship between species. A 3-genome comparison tool, called TaxPlot, provides a genome-wide approach to the study of gene and protein functions. TaxPlot produces a 2D plot in which the predicted proteins of a query organism are represented as points plotted with cartesian coordinates (X,Y) equivalent to best BLAST scores to predicted proteins from two other organisms. Another approach takes advantage of the COG (Clusters of the Orthologous Groups) system, which includes conserved protein families represented in at least three phylogenetically distant organisms with completely sequenced genomes. The proteins that comprise a COG are displayed in a whole genome graphical overview and are linked to the COG database.

The website will continue to evolve as more sequence enters the public domain, offering general display and analysis tools for all sequences, as well as resources tailored for selected organisms or sequence types. The Entrez Genomes home page is at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Genome.

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